INVESTIGATION INTO THE PIGMENTATION AND MEMBRANE STRUCTURE OF SARCINA AURANTIACA

Elizabeth M. M. Gray

A Thesis Submitted for the Degree of PhD
at the
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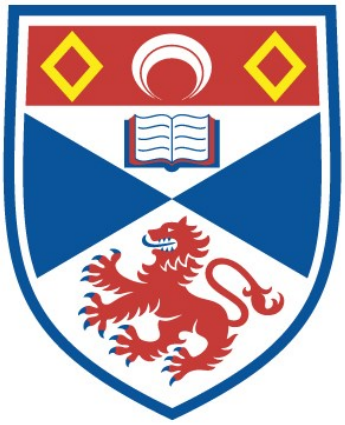

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INVESTIGATION INTO THE PIGMENTATION AND MEMBRANE STRUCTURE OF SARCINA AURANTIACA

being a thesis presented by

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to the University of St Andrews in application for the degree of Doctor of Philosophy

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DECLARATION

I hereby declare that the following thesis is based on work carried out by me, that the thesis is my own composition, and that no part of it has been presented previously for a higher degree.

The research was conducted in the Department of Biochemistry, United College of St Salvator and St Leonard, University of St Andrews, under the direction of Dr D. Thirkell.

CERTIFICATE

I hereby certify that Elizabeth M. M. Gray has spent nine terms engaged in research work under my direction and that she has fulfilled the conditions of the General Ordinance NO. 12 (Resolution of the University Court No. 1, 1967) and that she is qualified to submit the accompanying thesis for the degree of Doctor of Philosophy.

ACADEMIC RECORD

I first matriculated at the University of St Andrews in October 1965, and graduated with the degree of Bachelor of Science, Second Class Honours (Division I) in Biochemistry in June 1969. I matriculated as a research student in the Department of Biochemistry, University of St Andrews, in October 1969.

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INTRODUCTION

The generic name Sarcina was first proposed in 1842 when Sarcina ventriculi was isolated. According to Bergey (1957), the genera Sarcina, Staphylococcus and Micrococcus form part of the family Micrococcaceae. Classification of these Gram-positive, catalase-positive cocci has been very difficult. Early methods of classification involving morphology, physiological and biochemical reactions were not adequate (Berghey, 1957, Cowan, 1963, Baird - Parker, 1963). Baird - Parker (1965) also attempted classification using the chemical components of the cell, e.g. amino acids, hexoses, hexosamines and proteins, as taxonomic criteria but again this method was only partly successful.

Rosypal, Rosypalova and Horejs, (1966), and Bohacek, Kocur and Martinec, (1967) studied the guanine and cytosine (G + C) content in the DNA of strains of Micrococcaceae. Bohacek et al (1967) found that the staphylococci contained a low G + C content of 30.7 - 36 moles per cent and the Micrococci had a high value of 66.3 - 73.3 moles per cent. If the mean G + C content of the DNA differed by 10% or more the organisms were said to be phylogenetically distinct (Sueoka, 1961; Rosypal et al, 1966). Using a combination of the above taxonomic criteria, the Micrococcaceae were divided into two main groups:-

Staphylococci (facultative anaerobes) and Micrococci (aerobes and facultative anaerobes).

Initial classification of the genus Sarcina was mainly based on the mode of division of these organisms which divide regularly in three planes

forming a cubical bundle of eight (or more) bacteria. This characteristic was not sufficient on which to base a genus as physiological characteristics within the genus showed wide variations (Baird - Parker, 1965). This genus included the aerobic species S. lutea, S. flava and S. aurantiaca, anaerobic species S. maxima and S. ventriculi, the halophilic organism S. gigantea and the motile spore forming S. urea. Kocur and Martinec (1962) and Baird - Parker (1965) subsequently discovered that this packet formation depended on growth conditions for most species, although it was obligatory for the anaerobes S. ventriculi and S. maxima. Thus, packet formation was not a stable characteristic and therefore not a valid criterion for placing aerobic cocci in the genus Sarcina.

Kocur et al, (1962) suggested that the genus Sarcina should include only anaerobic cocci. The G + C content in the DNA of S. ventriculi and S. maxima (30.6 and 28.6 moles per cent) indicated that these organisms were phylogenetically remote from aerobic strains which contained 68 - 74 moles per cent G + C. Canale-Parola (1970) stated that aerobic, non-sporeforming, non-motile, packet-forming cocci which have a 68 - 74 moles per cent G + C in the DNA were indistinguishable from micrococci both physiologically and with respect to G + C content of the DNA. S. aurantiaca (ATCC 146) has a G + C content of 68.0 (Rosypal et al, 1966) or 72.8 (Kocur - private communication, 1970) and now some authors refer to S. aurantiaca as a Micrococcus species (ATCC 146).

Protoplast membranes have been isolated from several Gram-positive

bacteria since the initial isolation of membranes from lysed protoplasts of Bacillus megaterium, (Weibull, 1953). Membranes have been isolated by the use of muralytic enzymes e.g. lysozyme (mucopeptide-n-acetyl muramyl hydrolase E.C. 3.2.1. 17) which digest the outer cell wall of bacteria. If the digestion is performed in isotonic medium, protoplasts are formed. The protoplasts are then disrupted by osmotic lysis in a hypotonic medium followed by centrifugation and finally washed to remove the cell wall and cytoplasmic contaminants. (Salton and Freer, 1965b; Hunter, 1971).

The purity of membrane fractions has been checked by chemical analysis for cell wall contamination e.g. hexosamines, muramic acid and/or by examination of the membrane preparations under the electron microscope. The possibility of contamination of the membrane preparation with lytic enzymes (Shockman, Kolb, Bakay, Conover and Toennies, 1963; Panos and Cohen, 1966) giving an elevated protein content can be avoided by using a minimum quantity of enzyme. The cytochromes and carotenoids of many Gram-positive bacteria are specifically localised in the membrane (Salton and Ehtisham-ud-din, 1965a) and the stability of membranes during isolation can be checked by observing any loss of these membrane markers during the isolation procedure.

The membrane system of Gram-positive bacteria consists of two interrelate structures, the plasma membrane and mesosomes (intracytoplasmic structures) although the latter have not been detected in all Gram-positive bacteria.

Mesosomes arise from a process of invagination of the plasma membranes. The above methods of membrane isolation do not differentiate between the two membrane systems. Recently, workers claim to have separated the plasma membrane and mesosomes by density gradient centrifugation (Ghosh and Murray, 1969; Patch and Landman 1971). They demonstrated that the two fractions showed only slight differences in chemical composition but had markedly different enzymatic activities.

The cytoplasmic membranes of Gram-positive bacteria have been reported to contain between 9 and 36% lipid, 40 and 75% protein, 0.2 and 20% carbohydrate and to represent between 9 and 49.6% of the dry weight of the cell. (Table I. 1.). Thus, the overall chemical composition of bacterial membranes is similar to that of membranes isolated from other sources, that is they are largely lipoprotein in nature. Electron micrographs show that the plasma membrane and mesosomes have the features of a unit membrane with an overall thickness of 75 to 100Å.

Chemical analysis of bacterial membranes of the same species have shown wide variation in protein, lipid, carbohydrate and phosphorus content (Table I. 1.). These variations are in some cases as large as those observed for unrelated organisms. These differences are mainly due to the following reasons :-

(a) methods used for membrane isolation and purification (Salton, 1967a; Ghosh and Carroll, 1968)

(b) analytical methods used (Salton, 1967b)

(c) growth conditions employed (Shockman et al, 1963; Salton et al, 1965b)

There is still relatively little known about the type of proteins found in bacterial membranes, even though at least 40% of the membrane consists of protein. The presence of various enzyme systems and permeases indicates that membrane protein is not a single homogeneous component (Gilby et al, 1958) and various workers have fractionated the membrane into several protein components. Salton (1967b) used disc gel electrophoresis and found a degree of species specificity in the number and position of protein bands. However, other workers (Baird - Parker, 1965) have detected little species specifity so that protein fractions are of doubtful use as taxonomic criteria. Whereas Estrugo, Larraga, Corrales, Duch and Munoz, (1972) fractionated the membrane proteins of M. lysodeikticus into a minimum of twelve components, according to Salton (1967a), the membranes of several Gram-positive bacteria probably contain a minimum number of twenty protein fractions.

Protein contents varying from 40 to 75% of the total organic material have been reported for bacterial membranes of various species and the variations are mainly due to the analytical methods employed e.g. Salton (1967a) reports values of 49% (Lowry method) and 64.5% (Biuret method) for the same batch of M. lysodeikticus membranes. Other methods such as total nitrogen and Moore and Stein can be even less reliable for membrane preparations, in that they are subject to contributions from other non-protein components.

Hunter, (1971) reported a decrease in protein content from 63.6

BACTERIUM	% PROTEIN	% LIPID	% CARBOHYDRATE	% RNA	% PHOSPHORUS	REFERENCE
S. aureas H	62.4 - 70.6	21.8 - 24.6	0.81 - 0.97	4.47 - 4.78	1.08 - 1.18	Ward and Perkins 1968
S. aureas 100	62.6 - 67	21.8 - 25.6	0.80 - 0.89	2.03 - 3.80	1.06 - 1.20	
S. aureas						
Plasma membranes	56	25	4	14	-	Theodore, Popkin
Mesosomes	41	34	4	8	-	and Cole 1971
M. lysodeikticus	50	28	15 - 20	Trace	1.16	Gilby, Few and McQuillen 1958
M. lysodeikticus	63 - 75	14.6 - 29.5	4	1.4 - 2.7	0.5 - 0.9	Salton, et al, 1965b
S. lutea	39.8	28.9	9.6	1.2	-	Brown, 1961
S. lutea	53 - 61	20.0 - 26.7	-	3.3 - 7.5	0.6	Salton, et al, 1965 b
S. flava	51.2 - 63.6	14.7 - 19.9	6.3 - 7.3 13.8 - 14.5	1.9 - 2.9	0.7 - 1.2	Hunter, 1971
S. faecalis	49 - 55	28	-	-	-	Shockman, et al, 1963
L. monocytogenes	55 - 60	30 - 35	1.2 - 2.3	1.5	3.5 - 4.0	Ghosh, et al, 1968
B. megaterium	70	25	-	-	-	Yudkin, 1966
B. licheniformis	75	28	-	0.8	-	Salton, et al, 1965 b

Table I 1

Chemical Composition of the Membranes of some Gram-positive Bacteria

to 51.2% in the membranes of S. flava from the exponential to the stationary phase of growth, and Shockman et al, (1963) also detected a decrease in the membrane protein content from the log phase to the stationary phase in a threonine depleted culture of S. faecalis. However, Salton et al (1965b) found comparatively little change in the protein content of M. lysodeikticus membranes between cells grown for 24 hours and for 48 hours in several media.

Gilby et al (1958); Shockman et al, (1963); Grula, Butler, King and Smith (1967); Ghosh et al, (1968); Ward et al, (1958) and Hunter (1971) have all published data for the amino acid composition of bacterial membranes. With the exception of the report by Grula et al, (1967), bacterial membranes have a high content of alanine, glycine, leucine and isoleucine, aspartic and glutamic acid residues and a low proportion of basic and sulphur amino acids. This is in accordance with observations for membranes isolated from higher organisms which have a high content of hydrophobic and acidic amino acid residues (Kodicek, 1962). This indicates that the major type of bonding is of the hydrophobic type and relatively little ionic bonding exists.

Grula et al, (1967) reported that membranes of M. lysodeikticus also contained a high concentration of alanine, glycine and leucine but differed from other workers in finding a high concentration of basic amino acids. They also found a high ammonia peak during amino acid analysis which suggested deamination of asparagine and glutamine.

It is believed to be fairly typical to obtain reasonably high ammonia peaks of this nature during amino acid analysis of bacterial membranes, (Thirkell, 1972). However, Grula et al, (1967) considered that the major type of bonding is hydrophobic but also suggested that the high amount of basic amino acid residues might be available for ionic binding with phosphate groups in phospholipids and nucleic acids. Some unusual components were found in the amino acid hydrolysates from L. monocytogenes membranes, (Ghosh et al, 1968) and were identified as phosphoserine and glyceryl phosphoryl ethanolamine.

Little information would seem to have been obtained concerning the effects of growth conditions on the amino acids in membranes. However, Shockman et al, (1963) found that S. faecalis membranes from cells grown on a threonine-limiting medium showed an increase in aspartic and glutamic acids and lysine with a corresponding reduction in the other amino acids by 20. 30%. Hunter (1971) reported that the molar ratios of individual amino acids from S. flava membranes varied little with the age of the culture. The only significant variation was an increase in aspartic acid and a decrease in glycine and alanine for a 57 hour culture in relation to the 24 hour and 91 hour cultures.

The amount of total carbohydrate in bacterial membranes has also shown wide species variation and differences within the same species (Salton, 1967b). This is, again, mainly due to the analytical methods employed and to the conditions used in the isolation of the membranes. The phenol-H_2SO_4 method of estimation of carbohydrate usually gives higher results than the anthrone method, the former approximating to

total carbohydrate (hexose + pentose) and the latter to total hexose. The phenol-H_2SO_4 method is not subject to interference from hexosamine or protein (Whistler and Wolfrom, 1962).

The methods of membrane isolation affect the quantity of carbohydrate found. Gilby et al, (1958) reported 20% carbohydrate (anthrone method) in M. lysodeikticus membranes, whereas, Salton et al, (1965b), working with the same species, reported a value of only 4% again using the anthrone method. Gilby et al, (1958) probably isolated a "mannan" with the membrane, and they also reported 3.7% hexosamine indicating cell wall contamination. Also, Weibull and Bergstrom, (1958) found that the carbohydrate values varied from 1 to 10% (anthrone method) when they investigated 4 separate batches of membranes from Bacillus megaterium. Any cell wall contaminant would obviously increase the carbohydrate value.

Table I. II gives a quantitative and qualitative analysis of the carbohydrates reported in various bacterial membranes. There is no species correlation for the individual monosaccharides in the whole membrane and thus carbohydrate analysis would be of no taxonomic value. Glucose is the most abundant monosaccharide found in several bacterial membranes. Ribose is probably a constituent of the nucleic acids of membrane-bound ribosomes (Salton et al, 1965b). The occurence of other pentoses is unusual and when present in the membrane they do not appear to be cell wall contaminants (Ghosh et al, 1968; Hunter, (1971).

Hexoses are bound in the membrane in combination with lipid, protein or as polysaccharides. In some species the glycolipid accounted for all the carbohydrate present e.g. S. aureas (Ward et al, 1968) but in other species e.g. L. monocytogenes (Ghosh et al, 1968) not all the membrane carbohydrate could be accounted for as constituents of the glycolipid. Other species also contain polysaccharides e.g. Gilby et al, (1958) suggested that the membrane mannose might be largely in the form of a mannose polymer in M. lysodeikticus and Weibull et al, (1958) found that a glycogen-like polymer formed part of the carbohydrate in the membranes of B. megaterium. Hexoses and occasionally pentoses are found as constituents of diglyceryl diglycerides, glycophospholipids and carotenoid glycosides. Monosaccharides have also been reported to link carotenoid to protein in the membrane (Thirkell and Hunter, 1969).

Hunter (1971) investigated the effects of age of culture on S. flava membrane carbohydrate. No significant variation with age was detected for either total carbohydrate or relative amounts of the individual monosaccharide present.

There is far more qualitative and quantitative information concerning lipids than any other membrane component. Reviews compiled by Kates (1964) and O'Leary (1967) are very extensive. Most types of bacterial lipids have been investigated including fatty acids, glycolipids, phospholipids, glycophospholipids, carotenoids and aliphatic

BACTERIUM	% CARBOHYDRATE IN MEMBRANES	HEXOSES	PENTOSES	REFERENCE
B. licheniformis	-	Glucose Galactose	-	Salton et al, 1965b
B. megaterium	1 - 10% A	Glucose	-	Weibull et al, 1958
B. steaothermophilus	-	Glucose	-	Salton et al, 1965b
M. lysodeikticus	20% A	Mannose	-	Gilby et al, 1958
M. lysodeikticus	4% A	Glucose Galactose Mannose	ribose	Salton et al, 1965b
S. lutea	-	Glucose Galactose Mannose	ribose	Salton et al, 1965b
S. flava	6.3 - 7.3%A 13.8 - 14.5%P	Mannose Glucose	ribose rhamnose	Hunter, 1971
S. aureas	0.81-0.97%P	Glucose	ribose	Ward et al, 1968
L. monocytogenes	1.2 - 2.3%	Glucose Galactose	ribose arabinose	Ghosh et al, 1968

where A = Anthrone method
P = phenol-H_2SO_4

TABLE I. II

Carbohydrate Composition of the Membranes from some Gram-positive Bacteria

hydrocarbons. The effects of growth conditions on lipid composition have been studied and taxonomic classification attempted with regard to lipid composition.

Lipids are specific constituents of the cell membrane in Gram-positive bacteria (Macfarlane, 1961; Vorbeck and Marinetti, 1965a). The quantity of lipids in bacterial membranes is often related to medium composition (Shockman et al, 1963; Salton et al, 1965b) and to the age of the culture (O'Leary, 1967; Hunter, 1971). O'Leary (1967) stated that the lipid composition of many bacteria increased with age and Shockman et al, (1963) found that the lipid content of S. faecalis membranes increased from 28% in log phase to 36 - 40% in stationary phase. However, Hunter, (1971) reported that the lipid content decreased with age for S. flava membranes, although this may merely reflect an increase in the proportion of bound lipid with age thus making the extraction more difficult.

The relative amounts of the different lipid classes also varies with growth conditions and the age of the culture e.g. Joyce, Hammond and White, (1970) reported a 15 to 20% increase in phospholipid between the exponential and stationary phases of growth of S. aureas. Kates, (1964) states that the amino esters of phosphatidyl glycerol tend to accumulate in the stationary phase of growth of many Gram-positive bacteria. Minnikin, Abdolrahimzadeh and Baddiley, (1971) found that under conditions of phosphate starvation (approaching the stationary

BACTERIUM	% LIPID	FREE LIPID	BOUND LIPID	NEUTRAL LIPID	GLYCOLIPID	PHOSPHOLIPID	REFERENCE
L. monocytogenes	30 - 35% A	-	-	15 - 20%	← 80 - 85% →		Ghosh et al, 1968
S. aureas H	21.8 - 24.6%	-	-	17.6%	10.3%	68.0%	Ward et al, 1968
S. aureas 100	21.8 - 25.6% A	-	-	15%	8.1%	69.2%	
S. aureas	2.5% B	2.5%	-	16%	← 84% →		Macfarlane, 1961
M. lysodeikticus	3.7% B	3.7%	-	18%	← 82% →		Macfarlane, 1961
M. lysodeikticus	28% A	-	-	21%	-	79%	Gilby et al, 1958
S. lutea	1.55%B	83.9%	16.1%	73.6%	← 22.7% →		Huston and Albro, 1964
S. flava	14.7 - 19.9% A	37 - 40%	60 - 62.8%	-	-	-	Hunter, 1971
A. crystallopoietes	4 - 5% B	-	-	20%	25%	55%	Shaw and Stead, 1971
M. laidlawii	8 -12% B	-	-	← 50% →		50%	Smith, 1969

% lipid :-
expressed as % cell membrane :- A
expressed as % dry cell wt :- B

TABLE I. III

Lipid Composition of the Membranes from some Gram-positive bacteria

phase of growth), B. subtilis W23 and B. cereus T will replace acidic phospholipids with acidic glycolipids.

Neutral lipids, glycolipids and phospholipids have been analysed to determine any phylogenetic relationships. Morrison, Tornabene and Kloos, (1971) studied the neutral lipids of some species of the family Micrococcaceae and found that all Gram-positive Micrococci (with one exception) contained qualitatively similar hydrocarbons. This division into two groups coincided with the division based on DNA composition (Bohacek et al, 1967). According to Morrison et al, (1971) the hydrocarbon composition of Micrococcus species ATCC 146 was approximately 20% of the total cell lipid, C_{25} hydrocarbon predominating. In addition Shaw and Baddiley, (1968) found that members of the same genus contain identical glycolipids. Membranes also show a high degree of species specificity as to the major type of phospholipid present (Salton, 1967c).

There are a greater variety of unusual fatty acids in bacteria than in higher organisms, e.g. hydroxy, branched methyl and cyclopropane fatty acids are all frequently encountered. Fatty acid patterns can be markedly affected by the composition of the growth medium, oxygen availability, pH and the age of the culture (O'Leary, 1962). Lipids tend to contain more unsaturated fatty acids at lower temperatures (Kates, 1964) and in some species, limitations in the media also affect the degree of unsaturation of the fatty acids (O'Leary, 1967). The

branched chain amino acid composition of the medium can also specifically affect the synthesis of branched chain fatty acids in some bacteria (Tornabene, Bennett and Oro, 1967; Kaneda, 1971).

Many workers have reported large and small variations in the fatty acid ratios with the phase of growth but other workers have detected very little alteration. For example, Ray, White and Brock, (1971a) working with Thermus aquaticus and Tornabene et al, (1967) in their study of the fatty acids of S. lutea found that the fatty acid composition varied little with time. Marked variations in the fatty acid composition with the phase of growth have been observed for B. cereus (Kaneda, 1971), S. flava (Hunter, 1971) lactobacilli strains (Veerkamp, 1971) and a Thermophilic bacillus species (Daron, 1970).

Akashi and Saito (1960) working on an unspecified species of Sarcina were the first to detect the anteiso branched C_{15} fatty acid now known to be common in Gram-positive microorganisms and these workers named it sarcinic acid (12 methyl tetra-decanoic acid). Morrison et al, (1971) have analysed the fatty acid content of several species of Staphylococci and Micrococci including Micrococcus Sp. (ATCC, 146), which was grown in trypticase soy broth to early stationary phase at 25°. They found that the fatty acids (greater than 3 moles per cent) were within the range C_{13} - C_{16}, the iso and anteiso C_{15} fatty acids predominating. They also stated that the fatty acids of

this Micrococcus species were similar to the fatty acids in three strains of M. roseus. However, Girard, (1971) found that another strain of M. roseus had a large quantity of straight chain saturated and unsaturated fatty acids in addition to some branched chain fatty acids. Tornabene et al, (1967) also found a different fatty acid pattern in S. lutea from that previously reported by Huston et al, (1964) and Cho and Salton, (1966).

The first attempt at correlating fatty acid composition with classification of bacteria was made by Abel, Schmertizing and Peterson, (1963). These workers found that members of the same family had qualitatively similar fatty acids but there were quantitative differences with different growth conditions. A correlation was found between Gram stain and fatty acid composition but there are exceptions which make the correlation unreliable. (Abel et al, 1963; Girard, 1971).

Gram-negative bacteria tend to have even numbered, saturated and unsaturated straight chain fatty acids together with cyclopropane fatty acids, whereas Gram-positive bacteria have mainly odd numbered branched chain fatty acids and a relatively low amount of straight chain or unsaturated fatty acids (Kates, 1964). Anaerobic bacteria tend to have a high proportion of straight chain, saturated and unsaturated C_{16} acids (Cho et al, 1966) and Gram-variable bacteria tend to have a mixture of the two characteristics e.g. M. roseus and

M. radiodurans (Girard, 1971). Exceptions to the above generalisations are the lactobacilli and clostridia which have fatty acids resembling those from Gram-negative bacteria (Kates, 1964).

The Micrococcaceae, Bacillus and Streptomyces families all have similar fatty acid patterns which in turn differ from other bacteria and these families must therefore be closely related phylogenetically (Ueta and Yamakawa, 1968). Since many factors can be evidently influence the fatty acid patterns found in bacteria, growth conditions must be controlled or rigidly defined if fatty acids are to be of value in taxonomy, (Tadayan and Carrol, 1971; Hunter and Thirkell, 1971).

Hunter et al, (1971) found that the major fatty acid of the free lipid from S. flava was a saturated, branched chain C_{15} but the major fatty acid from the hydrolysed, bound lipid was a saturated straight chain C_{17}. However, Huston et al, (1964) found no significant differences in the fatty acid patterns between the free lipid and the hydrolysed, bound lipid of S. lutea.

Shaw (1970) reported that the fatty acid components of the glycolipids did not differ much from those of the phospholipids in Gram-positive bacteria, one exception to this rule being Staphylococcus lactis.

The small amounts of RNA found in bacterial membranes appear to be a specific membrane component (membrane bound ribosomes) since Ghosh et al, (1968) found that RNA was firmly bound to the membranes and

and could only be removed with RNase treatment. Varying amounts of RNA were found in cell membranes as Salton et al, (1965b) reported values of 0.8 to 7.5% and figures in the same range were obtained by Yudkin (1962) and Ward et al, (1968).

Ward et al, (1968) found that the RNA content of S. aureas H (~ 4%) was double the RNA in S. aureas 100 even though conditions of isolation and purification were identical. They suggest that this RNA discrepancy reflects a difference between two staphylococcal strains. Theodore et al, (1971) reported a value of 14% for the RNA in the plasma membranes of S. aureas (ATCC 6538P) and 8% for the mesosome fraction. The higher value of 14% is probably because membrane bound ribosomes have been shown to be preferentially associated with the plasma membrane in this organism.

Salton et al, (1965b) found that the RNA composition of M. lysodeikticus membranes increased slightly from 24 hours to 48 hours in three different media, whereas Hunter (1971) detected a change in the RNA composition of S. flava membranes throughout the growth cycle. However, Ghosh et al, (1968) consider that there is the possibility of contamination of the membrane fraction with cytoplasmic RNA and this should be borne in mind particularly when considering the variations of RNA which have been reported.

The amount of phosphorus detected in Gram-positive bacterial membranes is up to 4% (see Table I. 1) and both Gilby et al, (1958)

and Ward et al, (1968) showed that 60 -70% of the total phosphorus was generally associated with the lipid components. However, Ghosh et al, (1968) found that all of the phosphorus present in the membranes of L. monocytogenes could not be accounted for by the lipid and nucleic acid phosphorus alone, and suggested that the remainder might be associated with phosphoserine (detected in protein hydrolysates) or in membrane teichoic acids.

The amount of phosphorus in the membrane fraction may or may not be stable during growth since Salton et al (1965b) reported little variation in the percentage of phosphorus in the membranes of M. lysodeikticus grown for 24 hours and 48 hours, whereas Hunter (1971), detected a slight decrease in the membrane phosphorus level in S. flava from the exponential to the late stationary phases of growth.

Teichoic acids (polyol phosphate polymers) are important for the maintenance of cellular activity. Wall teichoic acids are common but not obligatory for Gram-positive bacteria whereas membrane teichoic acids appear to be always present in Gram-positive bacteria (Heptinstall, Archibald and Baddiley, 1970). All membrane teichoic acids are glycerol phosphate polymers having D-alanine ester residues and glycosyl substituents. Appreciable amounts of membrane teichoic acids are found in Gram-positive bacteria, although they are sometimes absent from membranes prepared from protoplasts. According to Heptinstall et al, (1970) membrane teichoic acids occupy a region between the cell

wall and the membrane so it is not clear to what extent these teichoic acids are true membrane components.

A lipid-teichoic acid complex has been isolated from the cytoplasmic membrane of S. faecalis WCB 8191 (Toon, Brown and Baddiley, 1972) which involved a covalent linkage between teichoic acid and glycolipid. About 12% of the membrane glycolipid was associated with this teichoic acid.

The presence or absence of teichoic acids from membrane preparations will obviously influence the qualitative and quantitative analysis for phosphate, sugars and alanine and may well be responsible for some of the discrepancies reported for these analyses.

The rapid increase in the knowledge of carotenoids is mainly due to the use of new separation techniques and methods of analysis. The use of infra red, visible and ultraviolet spectroscopy, mass spectrometry, NMR, PMR, and optical rotatory dispersion has aided the identification and the elucidation of the structures of carotenoids. As a result, whereas 15 carotenoids had been isolated by 1933, this number increased to about 80 in 1948 and by 1970 more than 300 carotenoids were known. The first major review on carotenoids was compiled by Karrer and Jucker (1948). Reviews by Goodwin (1965) and Liaaen-Jensen and Jensen (1965), and monographs by various authors were produced and most recently in 1971, a comprehensive multi-author review was compiled (Isler, 1971).

Carotenoid production in non-photosynthetic bacteria can be affected

by a number of environmental factors i.e. temperature, pH, oxygen, ionic strength and light. The effects of these factors varies with the organism and type of pigments produced (Thierry and Cooney, 1966). According to Goodwin (1963), most pigment producing, non-photosynthetic bacteria tend to produce more carotenoids at a lower temperature than that which is optimum for growth. Thirkell, Strang and Carstairs (1965) found that this was true for S. flava which had an optimum growth temperature of 30-35^{o} and an optimum pigmentation temperature of 26^{o}. However, M. roseus (Thierry et al, 1965) and M. radiodurans (Thirkell, 1969) appear to have the same optimum for growth and pigmentation.

Carotenoid synthesis can continue after the exponential phase of growth. Hammond and White (1970) found that during the aerobic growth cycle of S. aureas there was a 22-fold increase in the concentration of carotenoids in stationary-phase cells as compared with exponential-phase cells. The relative quantities of the individual pigment fractions also varied since the rubixanthins increased from 35% of the total pigment in the exponential phase to 75% in the stationary phase of S. aureas. Thirkell et al, (1965) reported that the pigmentation in S. flava reached an early maximum at 18 hours (exponential phase) which was maintained until 75 hours and then decreased slightly.

Oxygen has a marked effect on carotenoid synthesis since anaerobically grown cells of S. aureas contain negligeable quantities

of polar carotenoids (Hammond et al, 1970). In two halophilic cocci, oxygen is thought to play a role in the formation of a carotenoid glycoside (Aasen, Francis and Liaaen-Jensen, 1969). According to Goodwin (1971) the requirement for light in carotenoid synthesis depends on the species of bacteria.

The location of the carotenoid pigments has been determined in a few non-photosynthetic bacteria. In S. lutea and M. lysodeikticus (Mathews and Sistrom, 1959a; Salton et al, 1965a) and in S. flava (Strang, 1968) the carotenoids are specifically located in the cytoplasmic membrane. M. radiodurans (Work, 1964; Work and Griffiths, 1968) and the "Knallgas" bacterium 12/60/71 (Eberhardt 1971) are the only two bacteria found to have the bulk of their carotenoids associated with the cell wall. However, these two bacteria are not typical Gram-positive organisms since they contain lipid in the cell envelope.

The only two definite functions so far attributed to bacterial carotenoids are photoprotection and as accessory pigments in photosynthesis. Many other functions have been postulated but are only supported by inconclusive evidence (Krinsky, 1971).

Photoprotection against lethal photosensitization was first demonstrated in 1955 (Griffiths, Sistrom, Cohen-Bazire and Stanier) when a mutant of Rhodopseudomonas spheroides lacking carotenoid pigments was found to be photosensitive in the presence of air and light. Later, both Kunisawa and Stanier, (1958) and Mathews and Sistrom (1959b) showed

that carotenoids could also function as photoprotectors in non-photosynthetic bacteria. Mathews et al, (1965b, 1960) and Mathews (1963) proved that the carotenoids of S. lutea could protect cells against photooxidation catalysed by exogenous (e.g. toluidine blue) and endogenous photosensitizers in the presence of air and light. The endogenous photosensitizers were postulated to be the haem compounds normally present in the cell e.g. cytochromes. The carotenoids of S. lutea protect the enzymes and cytochromes of the respiratory chain by absorbing potentially harmful light energy (Mathews et al, 1959b).

Mathews et al, (1960) reported an increase in the membrane permeability during photodynamic killing but were able to prove that this was not the primary lethal effect. Photoprotection by carotenoids was proved to depend on the number of double bonds in the conjugated system (Stanier, 1959; Crounse, Feldman and Clayton, 1963). The minimum number of double bonds required was found to be nine (Roth and Krinsky, 1970a) but ten or eleven double bonds confered greater protection. Both Kunisawa et al, (1958) and Mathews and Krinsky, (1965) have shown that the carotenoids confer no protection against UV light or against X rays and γ rays in radiation-resistant bacteria.

Joyce, et al, (1970) found that cultures of S. aureas preferentially synthesize polar xanthophylls (rubixanthins)rather than carotenes at lower than optimum temperatures and they suggest that

rubixanthins have a similar function to unsaturated fatty acids in allowing membranes to function at sub-optimal temperatures. Original suggestions by Salton et al, (1965b) and by Salton et al, (1965a) that carotenoids are involved in stabilizing the cell membrane have been refuted by Roth and Krinsky (1970b) who could find no difference in osmotic fragility between the wild type and two carotenoidless strains of S. lutea. The suggestion by Smith (1963) that carotenoids can function as transporters of glucose into cells of Mycoplasma laidlawii has been disproved by Razin and Rottem (1967).

The ecological importance of carotenoids due to their functions as photoprotective agents suggests that all aquatic and asporogenous bacteria might contain carotenoids if exposed to light and air (Mathews et al, 1959b). It is thus interesting that carotenoids are also found in thermophiles e.g. T. aquaticus (Ray, White and Brock, 1971b) and radiation resistant microorganisms e.g. Micrococcus Radiodurans (Thirkell, 1969).

In several bacteria, carotenoid complexes are bound in the membrane and are not released by conventional extraction procedures, and consequently, more rigorous extraction methods are often required to recover this fraction of the membrane components. Thus, with the advent of new isolation techniques e.g. Ultrasonication and new detection techniques e.g. Mass spectrometry, PMR, carotenoid glycosides and glycopeptides have been detected in organisms previously thought to

contain only free pigments.

Naturally-occurring true carotenoid glycosides were unknown until Smith (1963) isolated a neurosporyl glucoside from Mycoplasma. Carotenoid glycosides were first considered to be rare but have now been isolated from blue-green algae, photosynthetic and non-photosynthetic bacteria (Hertzberg and Liaaen-Jensen, 1969a). Glycosides of true carotenoids have not been isolated from eucaryotic organisms and appear to be characteristic of procaryotes (Reichenbach and Kleinig, 1971).

Structures have been assigned to more than 14 carotenoid glycosides of C_{40} xanthophylls, two glycosidic apo-carotenoids and two glycosidic C_{50} carotenoids (Liaaen-Jensen, 1971). Some of these carotenoid glycosides are presented in Table I. IV. Structural elucidation has benn mainly due to PMR and Mass spectrometry of the acylated glycosides. Carotenoid glycosides are very polar and therefore are often best purified as acetates. Analysis of the sugar moiety and isolation of the aglycone in a modified form have aided identification but surprisingly, enzymatic hydrolysis of the carotenol-carbohydrate linkage has not been successful (Hertzberg and Liaaen-Jensen, 1967).

Most carotenoid glycosides isolated are assumed to be β glycosides in common with all the other naturally occurring glycosides and an instance of carotenoid linked to two sugar moietes by a hydroxyl group at either end of the molecule have been reported (Hertzberg et al, 1969a). Many of the carotenoid glycosides isolated are tertiary glycosides and

all the other C_{40} glycosides are secondary glycosides (Liaaen-Jensen, 1971). The only primary glycosides which have been isolated are the C_{50} glycosides.

The sugar moieties also vary considerably as glucosides, O-acyl glucosides (glucose esterified to fatty acids), mannosides, rhamnosides and O-methyl-5-C-methyl pentosides have been isolated (Liaaen-Jensen, 1971). A mixed carotenoid glycoside, myxoxanthophyll, has been isolated from blue-green algae (Hertzberg and Liaaen-Jensen, 1969b). In myxoxanthophyll, rhamnose is the major sugar component and a hexose is a minor component, both glycosides having the same aglycone, myxol in common.

The aglycones of carotenoid glycosides are related to the free pigment found in the organism. In fact, carotenoid glycosides probably represent the terminal product of the sequence beginning with the biosynthesis of free carotenoids. Unlike the caroteno-proteins (see below) from invertebrates the association of carotenoid with other molecules does not significantly alter the chromophore from that of the free pigment. In many organisms the carotenoid glycosides are a major fraction of the total carotenoid pigment (see Table I. IV).

The only carotenoid glycoprotein complex to be characterised was isolated from S. flava by Thirkell et al, (1969). The carotenoid molecule appears to be bound to glucose via a glycosidic linkage involving the reducing group on the sugar moiety. Peptides are assumed

SOURCE ORGANISM AND TRIVIAL NAME	CAROTENOID AGLYCONE	% TOTAL PIGMENT	SUGAR MOIETY	REFERENCE
Mycobacterium phlei phleixanthophyll	1',-2'-dihydroxy-1',2'-dihydro-3',4'-didehydro- δ -carotene (C_{40})	64%	D. Glucose	Hertzberg et al, 1967
Mycobacterium phlei 4-keto-phleixanthophyll	1',2'-dihydroxy-1',2'-dihydro-3',4'-didehydro-4-keto- δ -carotene (C_{40})	21%	D. Glucose	Hertzberg et al, 1967
Arthrospira Spp. Myxoxanthophyll	1',2',-dihydro-3',4'-didehydro 3,1',2' trihydroxy- δ -carotene (C_{40})		L. rhamnose + trace of hexose	Hertzberg et al, 1969b
Arthrospira Spp. Oscillaxanthin	1,1',2,2'-tetrahydroxy-1,2,1',2'-tetra-hydro-3,4,3',4' tetra dehydro lycopene (C_{40})	5%	L. rhamnose	Hertzberg et al, 1969a
Corynebacterium fasciens P. 462	1'-(?)-hydroxy-5-demethyl-2-methyl-1,2,3,4-tetrahydro δ carotene (C_{40})	15.7%	D. Glucose	Prebble, 1968
Sarcina lutea No trivial name	2,2'-Di-(2-hydroxy methyl but-2-enyl)-5'-hydroxy methyl ϵ carotene (C_{50})	20%	D. Glucose	Norgard, Francis, Jensen-Jensen, 1970
Corynebacterium Sp. NCMB8 Corynexanthin	2,2'-Di-(2-hydroxy methyl-but-2-enyl)- ϵcarotene (C_{50})	-	D. Glucose	Weeks and Andrews, 1970

Stigmatella aurantiaca Myxobactin	1'-hydroxy-2'-hydro-3,4,3',4' tetra-dehydro- β -carotene (C_{40})	80%	D. Glucose + fatty acids	Kleinig, Reichenbach and Achenbach,	1970
Stigmatella aurantiaca Myxobacton	4 keto-1'-hydroxy-2'-hydro-3', 4'-didehydro- β -carotene (C_{40})		D. Glucose + fatty acids	Kleinig, et al,	1970
Halophilic coccus, SE20-4	1-hydroxy-1,2-dihydro-3,4-didehydro-apo-8'-lycopene (C_{40})	64 %	D. Mannose	Aasen et al,	1967

TABLE I. IV

Some naturally occurring carotenoid glycosides

to be linked to free hydroxyl groups on the glucose moiety.

There is little detailed information about the very polar carotenoproteins (Weedon, 1971). They are mainly found in invertebrates but have been isolated from blue-green algae and a few non-photosynthetic bacteria (Haidak, Mathews and Sweeney, 1966; Saperstein and Starr, 1955; Mathews et al, 1959a). Carotenoproteins in invertebrates involve a stoichiometric linkage between protein and carotenoid which alters the chromophore considerably. Carotenoproteins and carotenoid glycosides are more stable to isomerisation, oxidative and hydrolytic agents than the free pigments (Cheeseman, Lee and Zagalsky, 1967).

Several theories have been proposed as to the configuration of the carotenoid in the membrane. According to Grula et al, (1967) most of the phospholipids and carotenoids of M. lysodeikticus are present as superstructures surrounding the protein structure of the basal membrane continuum. Smith (1969) proposed a model by which apolar segments of carotenoids are bound to the hydrophobic areas of the protein in the lipoprotein subunit. In this model, a di-hydroxy carotenoid would lie across the subunit, one hydroxyl pointing to the interior and one hydroxyl to the exterior of the cell. Therefore one hydroxyl could be available for the covalent attachment of glucose and this formed the basis of his proposal that carotenoids might be instrumental in glucose transport.

Thirkell et al, (1969) proposed a very similar model involving

the carotenoid glycopeptide from S. flava. Only in this case, there was no mention of hydrophobic interactions between carotenoid and protein. They stated that the carotenoid straddles the lipid layer of a bimolecular leaflet type of membrane, both hydroxyls covalently linked to glucose moieties. The glucose molecules are in turn linked to the protein subunits which are integral parts of the protein layers.

Schmidt, Pfennig and Liaeen Jensen (1965) found that classification based on criteria of carotenoid composition is of little value as species of the same genera can produce different carotenoids whereas bacteria from different genera can contain similar carotenoids. This is substantiated by the observations of Strang and Thirkell (1969) who showed that S. aurantiaca synthesizes different pigments from the otherwise closely related species S. flava, S. lutea and M. lysodeikticus.

There has been very little published concerning the carotenoids of S. aurantiaca. Reader (1925) reported β-carotene and lycopene and Chargaff (1933) claimed to have found β-carotene and zeaxanthin in this microorganism. Sobin and Stahly (1942) isolated nine pigments from S. aurantiaca including one carotenol not reported previously. They also noted the absence of esters and carotenoid acids. Strang et al, (1969) intimated that β-carotene and zeaxanthin were present in S. aurantiaca but gave no evidence to support this assumption.

AIMS OF THIS THESIS

The main lines of investigation of this work were as follows:-

1. To investigate the growth and pigmentation of S. aurantiaca at various temperatures and pHs.
2. To compare the chemical composition of the total membrane fractions of S. aurantiaca at different phases of growth with particular reference to the lipids.
3. To isolate and characterise the free carotenoids and any polar carotenoid glycopeptides which may occur in S. aurantiaca.
4. To elucidate the composition and properties of any water-soluble carotenoid glycopeptide fractions which could be isolated from S. aurantiaca.

METHODS

The Morphology of S. aurantiaca Flügge (ATCC 146)

The morphology of S. aurantiaca was checked periodically by Gram-staining and examination under the oil-immersion lens of the light microscope.

Maintenance Culture of S. aurantiaca

S. aurantiaca was grown on nutrient agar (Oxoid Ltd) + 2% glycerol (w/v) at 30°. A single colony was transferred to a sterile agar plate at weekly intervals.

Growth of S. aurantiaca was limited and clumping occurred in liquid and solid cultures unless 2% glycerol (w/v) was included in the medium (Sobin et al, 1942).

Small Scale Culture of S. aurantiaca

Bacterial cultures of up to 500 ml in volume were grown in a New Brunswick G_{25} orbital incubator shaker. This apparatus was used for growing inocula for the 12 litre cultures and also for investigating the optimum conditions for growth and pigmentation.

Large Scale Culture of S. aurantiaca

(a) Cells were grown in a New Brunswick MF 114 Microferm Bacterial Fermentor at 28° in 12 litres of nutrient broth (Oxoid Ltd) + 2% glycerol (w/v). The cultures were aerated at a rate of 12 litres per minute and the stirrer speed was 400 rpm. Cells

were harvested by centrifugation at 600g.

(b) Cells were grown at Imperial College London in 400 litres nutrient broth (Oxoid Ltd) + 2% glycerol (w/v) at 30°. The air flow rate was 400 litres per minute and the stirrer speed was 185 rpm. The culture was allowed to grow for 27 hours when half was harvested and the remainder harvested after 57 hours growth. The cells were harvested on a Sharples continuous flow centrifuge and the cells were deep frozen for transport. The approximate yield of S. aurantiaca was 5.2 gm wet weight per litre.

Investigation of optimum conditions for growth and pigmentation

Determination of the optimum temperature for growth

1 litre conical flasks containing 500 ml nutrient broth (Oxoid Ltd) + 2% glycerol (w/v) were inoculated with 1 ml of the same bacterial cell population from a 24 hour inoculum grown at 30°. Bacterial cultures in quadruplicate were grown at 20°, 25°, 28°, 30°, 35° and 40° in a New Brunswick G_{25} orbital incubator-shaker at a constant shaking speed of 300 rpm.

Aliquots were withdrawn at set time intervals and the bacterial numbers estimated by:-

(a) Turbimetric readings at 600 nm on a Unicam SP 600 spectrophotometer against a medium blank. At 600 nm, there was no interference from pigments present in the organism. The aliquots were diluted (when necessary) with sterile medium to give a maximum optical density reading of 0.35.

This was necessary as the Lambert-Beer Law only applies at low concentrations of bacteria.

(b) Viable cell counting. Up to 7 serial 1/10 dilutions were made using sterile medium and 0.1 ml from each dilution was plated out on nutrient agar plates (Oxoid Ltd) + 2% glycerol (w/v). Colonies on the appropriate plates (between 20 and 200 colonies) were counted on a Gallenkamp colony counter after allowing growth for 48 hours at 30°.

Determination of the optimum pH for growth

Media were prepared at the following pH values - 4.4, 5.4, 6.4, 6.6, 7.2, 7.4, 8.1, 9.4 by titrating the normal medium with either HCl or KOH and the pH was checked after autoclaving. Duplicate 250 ml volumes of media at each pH were inoculated with 1 ml of a 24 hour inoculum culture and grown at 28° (the determined optimum temperature for growth) and otherwise under the same conditions as above. Aliquots were withdrawn at regular time intervals and the cell population was monitored by turbimetric reading at 600 nm.

Determination of temperature and growth period for optimum pigmentation

Bacteria were grown at 20°, 25°, 30°, 35° and 40° under the same conditions as those given for the determination of optimum growth conditions. Aliquots were withdrawn at set time intervals and the

cells were harvested by centrifugation at 600 g. The pellet was washed twice with distilled water, suspended in methanol and subjected to ultrasonication at maximum output (60 watts). Treatment was continued for five periods of two minutes, alternating with one minute cooling periods, the tube containing the bacterial suspension being situated in an ice bath. The suspension was made to 10% (w/v) methanolic KOH by the slow addition of solid KOH with stirring, gassed out with nitrogen and left to saponify in the dark at room temperature overnight. After centrifuging at 600 g the supernatants were made up to 20 mls with methanolic KOH and the absorption read at 438 mm (the λ_{max} of the total pigments in methanol - KOH) on a Unicam SP 600 spectrophotometer. The bacterial remnants were then dried to constant weight at 105°. The relative amount of pigment produced per unit weight of cell remnants was calculated.

Chemical Analyses of the Cell Membranes from S. aurantiaca

Cultures of S. aurantiaca were grown and harvested after the following periods of time:-

17 hours (mid-logarithmic phase)

27 hours (early-stationary phase)

57 hours (late-stationary phase)

All three cultures were monitored by turbimetric reading at 600 nm using a Unicam SP 600 spectrophotometer to determine the phase of growth

at harvesting.

The 17 hour culture was grown in a 12 litre fermentor whereas the 27 hour and 57 hour cultures were part of the same batch grown in a 400 litre fermentor. In both cases, medium composition, temperature and rate of aeration were identical. The only differences between the 17 hour culture and the other cultures were a slower stirrer speed and lack of any illumination for the 27 hour and 57 hour cultures.

The total membrane fraction was prepared from the three cultures using the method of Salton et al, (1965b)-see figure M. 1. The digestion period with lysozyme was increased from 1 hour (recommended by Salton) to 8 hours to ensure the complete removal of the outer cell wall.

Dry Weight Determination

The dry weight was determined by heating triplicate 0.100 gm samples of membranes from each culture to constant weight at 105^{o}.

Ash Determination

Triplicate 0.300 gm samples of membranes from each culture were slowly heated to 500^{o} and maintained at that temperature for 3 hours. After cooling, a few drops concentrated H_2SO_4 were added followed by heating at 700^{o} for 3 hours. The residue was then re-heated to constant weight.

Determination of total Phosphorus (Allen, 1940)

The following reagents were used:-

Fig. M. 1.

Flow Diagram for the preparation of the total membrane fraction from S. aurantiaca (Salton et al, 1965b)

Suspension of cell (20 mgm wet wt/ml) in 0.1M Tris buffer pH 7.5 treated with 150 µgm/ml of crystalline egg white lysozyme (Sigma Ltd), on a magnetic stirrer at 25° for 8 hours.

↓

lysed protoplasts

↓

treated with 30 µgm/ml DNAase on a magnetic stirrer at 25° for 1 hour. Centrifuged at 500g.

↓

Pellet of cell wall debris and whole bacteria (discarded)

Membrane suspension

↓

centrifuged at 35,000g for 40 minutes at 4°

↓

Colourless supernatant (discarded)

Orange membrane pellet

↓

Dispersed in 0.05 M Tris buffer, washed X 3 with 0.05 M Tris buffer, centrifuging at 35,000 g for 40 minutes after each washing.

↓

Washed membrane pellet

↓

Suspended in tap water and dialysed against running tap water for 24 hours. Dialysed against 3 changes of distilled water for 24 hours at 4°. Centrifugation as above.

↓

Purified total membrane fraction

↓

Freeze-dried

↓

Lyophilised total membrane fraction

(a) 10 N H_2SO_4 (AR)

(b) Amidol reagent (1 gm 2:4 diaminophenylhydrochloride in 100 cc 20% (w/v) sodium metabisulphite)

(c) 8.3% ammonium molydbate

A standard graph was prepared using a solution of AR KH_2PO_4, 1cc of which was equivalent to 100 µgm phosphorus, and which was serially diluted to give concentrations from 10 µgm to 100 µgm.

. 6 mgm samples (in triplicate) from each age of membrane were weighed into microkjeldahl tubes and hydrolysed by refluxing with 1.2 ml 10 N H_2SO_4. A few drops of 10 volumes H_2O_2 were added to decolourise the digest followed by further heating for 15 minutes. After cooling, the following reagents were added:-

6.4 cc water, 2 cc amidol, 1 cc ammonium molybdate and finally 15 cc water.

After standing for at least 10 minutes but not more than 30 minutes the optical density was read at 640 nm using a Unicam SP 600 spectrophotometer.

Determination of RNA (Schneider, 1957)

The following reagents were used:-

(a) orcinol reagent :- 1.0 gm orcinol dissolved in 100 ml concentrated HCl containing 0.4 g $FeCl_3$.

(b) Trichloroacetic acid (TCA).

A calibration curve was prepared using 0-100 µgm ribose.

500 mgm samples of each membrane preparation were soaked in a minimum volume of distilled water and extracted with 100 ml $CHCl_3$: MeOH (2:1) v/v by stirring for 3 hours. The defatted membranes were lyophilised and weighed.

100 mgm defatted membranes were suspended in 2.5 ml cold 10% (w/v) TCA, centrifuged and the pellet was re-extracted with 2.5 ml 10% TCA to remove the acid-soluble materials and both supernatants were discarded. The pellet was then suspended in 2.5 ml 5% (w/v) TCA and heated at 90° for 15 minutes with occasional stirring. After centrifuging, the pellet was re-extracted with 2.5 ml 5% TCA as above and centrifuged again. The combined supernatants (5.0 ml) contain the total RNA of the sample. This method depends on the preferential extraction of RNA by hot TCA.

Triplicate 0.2 ml aliquots of the RNA extracts from each membrane sample were diluted to 1.5 ml and heated with 1.5 ml of orcinol reagent for 20 minutes at 100°. Immediately after cooling, the optical density was read at 660 nm using a Unicam SP 600 spectrophotometer.

Protein Determination and Amino Acid Analysis

25 mgm samples (in duplicate) of each membrane preparation were hydrolysed in 6 N HCl in sealed tubes at 110° for 18 hours. The humin was filtered off, washed and the acid removed by azeotropic distillation with water. The acid-free hydrolysate was assayed for

protein content by the method of Moore and Stein (1954). The following reagents were used:-

(1) Acetate buffer pH 5.5:- 544 gm sodium acetate were dissolved in 400 ml warm water. After cooling, 100 ml glacial acetic acid were added and the volume made up to 1 litre.

(2) Ninhydrin reagent:- 1 gm ninhydrin + 150 mgm hydrintanin in 37.5 ml methoxyethanol + 2.5 ml acetate buffer.

Triplicate 1 ml samples of each membrane hydrolysate were shaken with 1 ml ninhydrin reagent and left at 100° for 15 minutes. After cooling, the samples were diluted with 5 ml 50% (v/v) ethanol and shaken for ½ minute. The absorbances were read at 570 nm using a Unicam SP 600 spectrophotometer. A standard graph was prepared using solutions containing 0-1 µmole / ml of nor-leucine.

The amino acid composition of the membrane hydrolysate was determined using a Locarte amino acid autoanalyser using nor-leucine as an internal standard.

Two methods for extracting proteins from S. aurantiaca membranes were employed:-

1. Method according to Maddy and Kelly (1970).

The details of this method are given in figure M. II.

(2) Method of Jeffrey (Private communication, 1971).

This procedure is shown in figure M. III.

F_A by virtue of its colour, consistency and mode of extraction is likely to contain a high proportion of lipid and therefore the protein is probably in the form of lipoprotein rather than as free protein.

Attempted resolution of the protein fractions from Methods 1 and 2.

(a) High voltage electrophoresis.

Fractions 1, 2 and 4 from Method I were applied to Whatman No. 1 paper and run at 3,000 volts for 45 minutes in acetate buffer (0.05M) at pH 4.75. The papers were dried at 100° and stained with ninhydrin.

(b) Disc-gel electrophoresis

Polyacrylamide gels were prepared as follows:-

Solution I : 48 ml 1 M KOH + 17.2 ml glacial acetic acid + 4.0 ml tetramethylethylenediamine (TEMED), made up to 100 ml with distilled water.

Solution II : 30 gm cyanogum 41 in 100 ml distilled water.

Solution III : 0.004 gm riboflavin in 100 ml distilled water.

The gel solution was prepared by mixing 1 volume solution I + 2 volumes solution II + 4 volumes solution III + 1 volume distilled water. After mixing, the gel solution was pipetted into the tubes, the gel overlayed with distilled water and polymerisation activated by a fluorescent strip light placed in front of the tubes.

Solutions of fraction 1, 2 and 4 (method I), F_A (method II) and bovine serum albumin standard (1mgm/ml) were made up in 5% (w/v) sucrose and applied to the top of the gels with a syringe. The buffer compartments of the apparatus (Shandon) were filled with 0.005 M acetate buffer, pH 4.75 and the runs made at a constant voltage of 300 volts for periods ranging from 1 hour to $2\frac{1}{4}$ hours. After careful removal of the gels at the termination of the runs, they were stained with 1% amido black in 7% acetic acid for 2 hours. Then, after washing the gels in distilled water, they were electrolytically destained in 7% acetic acid at 150 volts for 1 hour.

Carbohydrate Determination

Quantitative

25 mgm samples of each membrane preparation (in duplicate) were hydrolysed in 1 M HCl for 4 hours at 105°. The acid was removed as before and the total carbohydrate present was determined using the phenol-H_2SO_4 method (Whistler et al, 1962).

The following reagents were used:-

(a) AR 5% (w/v) phenol

(b) AR 96%(v/v) H_2SO_4

A standard graph was prepared using solutions containing 0-100 µg/ml of glucose. 1 cc aliquots, in duplicate, of each membrane hydrolysate were added to 1 cc 5% (w/v) phenol and mixed. 5 cc H_2SO_4 were added

FIG. M. II METHOD I (MADDY AND KELLY, 1970)

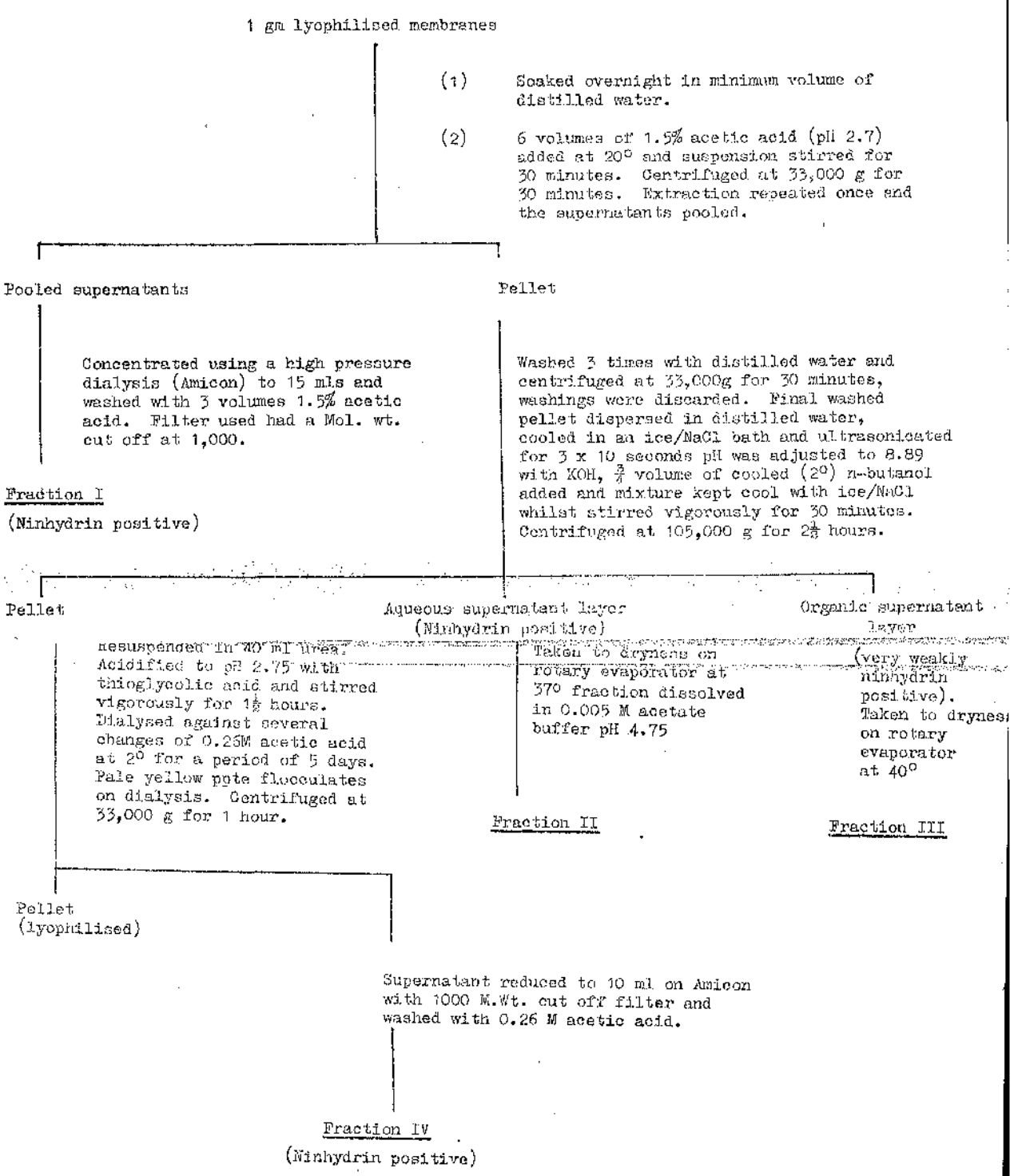
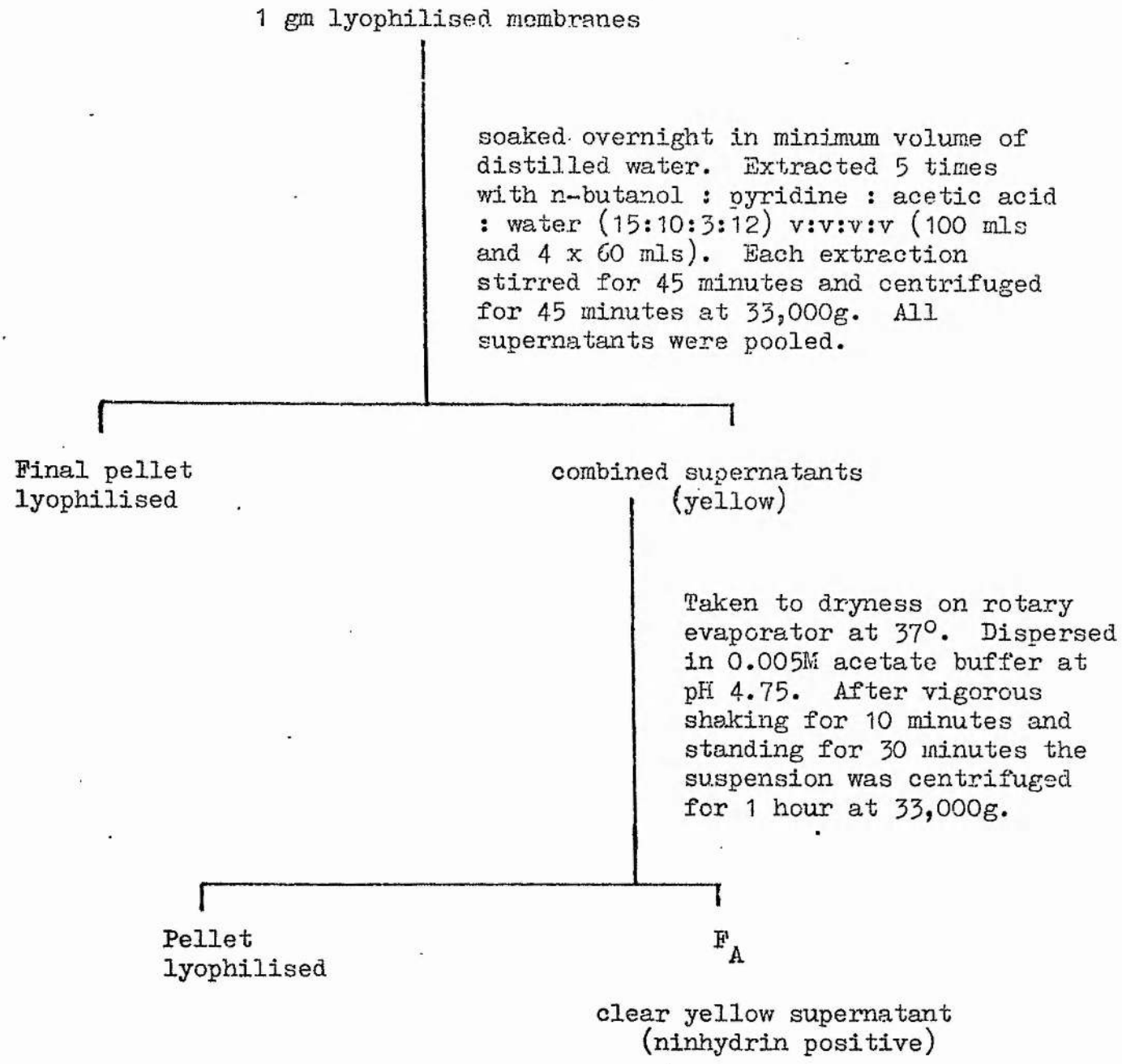


FIG. M. III METHOD II (JEFFREY, 1971)



quickly from a fast flowing burette, to ensure rapid mixing and the tubes were shaken. After 10 minutes, the tubes were reshaken and heated at 25-30° for 20 minutes. Using a Unicam SP 600 spectrophotometer absorbances were measured at 490 nm. The yellow-orange colour formed is stable for several hours.

An initial experiment using the phenol-H_2SO_4 method determined the optimum hydrolysis time required to produce a maximum release of carbohydrate from complexes but it must be realised that prolonged hydrolysis may lead to degradation of some hexoses liberated early on. Hydrolysis times up to 24 hours were assayed.

Qualitative

Paper Chromatography

Descending chromatograms of the concentrated hydrolysates from above were run on Whatman No. 1 paper using the following solvent systems:-

(1) n-butanol : acetic acid : water (6:1:2) v/v/v

(2) n-butanol : pyridine : water (6:4:3) v/v/v

(3) Ethyl acetate : pyridine : water (70:20:23) v/v/v

The standards used were galactose, glucose, mannose, ribose, arabinose, glucosamine hydrochloride and galactosamine hydrochloride.

Since the results obtained using paper chromatography were not

very conclusive, gas-liquid chromatography of the trimethysilyl ester derivatives of the membrane carbohydrates was employed.

Gas-liquid Chromatography (GLC)

10 mgm samples of membranes from each culture were hydrolysed in 1M HCl and made acid free as before. The following trimethyl-silylation reaction was deleloped by Sweeley, Bentley, Makita and Wells (1963).

Standard solutions (1 mgm/ml)were prepared of:-

galactose, glucose, mannose, ribose, arabinose, rhamnose, fucose, xylose, fructose, arabitol, ribitol, sorbitol, dulcitol, mannitol, glucuronic acid and N-acetyl glucosamine.

A solution of α-methyl-D-glucoside for addition to standards and samples as an internal standard was also prepared.

The samples and standards were completely dried in vacuo and dissolved in 1 ml dry, redistilled pyridine. 0.2 ml hexamethyl-disilazane and 0.1 ml trimethylchlorosilane were added. The reaction was complete after 30 minutes at room temperature.

A Pye model 104 Gas Chromatograph was used under the following conditions:-

Twin glass columns	:-	5 ft, 10%SE30 on 100-120 mesh celite
Column temperature	:-	185°, isothermal
Detector oven temperature	:-	210°
Flash heater	:-	230°
Attenuation	:-	10×10^2
Carrier gas (N_2) flow rate	:-	45 ml/min.

Air to detector	:-	600 ml/min.
H_2(to detector)	:-	40 ml/min.
Chart speed	:-	25 mm/min.

The retention times relative to the retention time of α -methyl-D-glucoside were calculated for the standards and for the hydrolysates. The trimethylsilylation reaction is quantitative and the amount of each sugar present was calculated by measuring the areas under the peaks. However, some difficulties arise with quantitation of a mixture of sugars due to overlapping of peaks. GLC gives an accurate, fast, qualitative analysis of the monosaccharides present.

The amino acid hydrolysates showed that negligible amounts of galactosamine and glucosamine were present in the membrane hydrolysates. It was decided to check for the presence of these hexosamines using GLC.

GLC of Hexosamines

Hexosamine hydrochlorides do not readily give volatile TMS derivatives unless N-acetylated prior to silylation. 25 mgm samples of membranes were hydrolysed in sealed tubes in 4N HCl at 100^0 for 8 hours. The hydrolysates were dried under vacuo. 100 µgm standard solutions of galactosamine hydrochloride and glucosamine

hydrochloride were also dried under vacuo. The following trimethyl-silylation procedure was developed by Stimson (1971). Hexosamines were trimethyl-silylated using bis (trimethylsilyl) trifluoroacetamide (BS TFA). BS TFA reagent consisted of 25% BS TFA in dimethylacetamide with phenanthrene as internal standard at a concentration of 40 µgm/0.1 ml.

0.1 ml BS TFA reagent was added to each dry sample and heated at 100^{o} for 10 minutes.

A Pye model 104 gas chromatograph was used under the following conditions:-

Single column	:-	5ft glass : 3% Apiezon L on 100-120 mesh celite
Column temperature	:-	175^{o}, isothermal
Detector oven temperature	:-	200^{o}
Flash heater	:-	220^{o}
Attenuation	:-	2×10^{2}
Carrier gas (N_2) flow rate	:-	45 ml/min.
Air (to detector)	:-	600 ml/min.
H_2 (to detector)	:-	40 ml/min.
Chart speed	:-	5 mm/min.

Retention times relative to phenanthrene were calculated for the standards and for the components of the hydrolysates.

Lipid Determination

Extraction

An increased yield of lipid was obtained when the lyophilised membranes were soaked in a minimum volume of distilled water prior

to extraction with organic solvents. Two methods were used for extracting lipid from S. aurantiaca membranes. The first method tried was a modification of the lipid extraction method of Huston et al, (1964) as used by Hunter (1971). See figure M. IV for details of Method I.

In the second method, 1 gm soaked lyophilised membranes were suspended in 150 ml $CHCl_3$: MeOH (2:1) v/v and stirred for 3 hours on a magnetic stirrer at room temperature. The suspension was filtered on Whatman No. 1 filter paper and the residue re-extracted twice. The residue was then extracted by refluxing with organic solvents as in the previous method.

The main advantage of the second lipid extraction method was the absence of heat in the first extraction steps and therefore the lipid was less liable to be degraded during isolation. Surprisingly, the second method extracted a larger quantity of lipid than the first method.

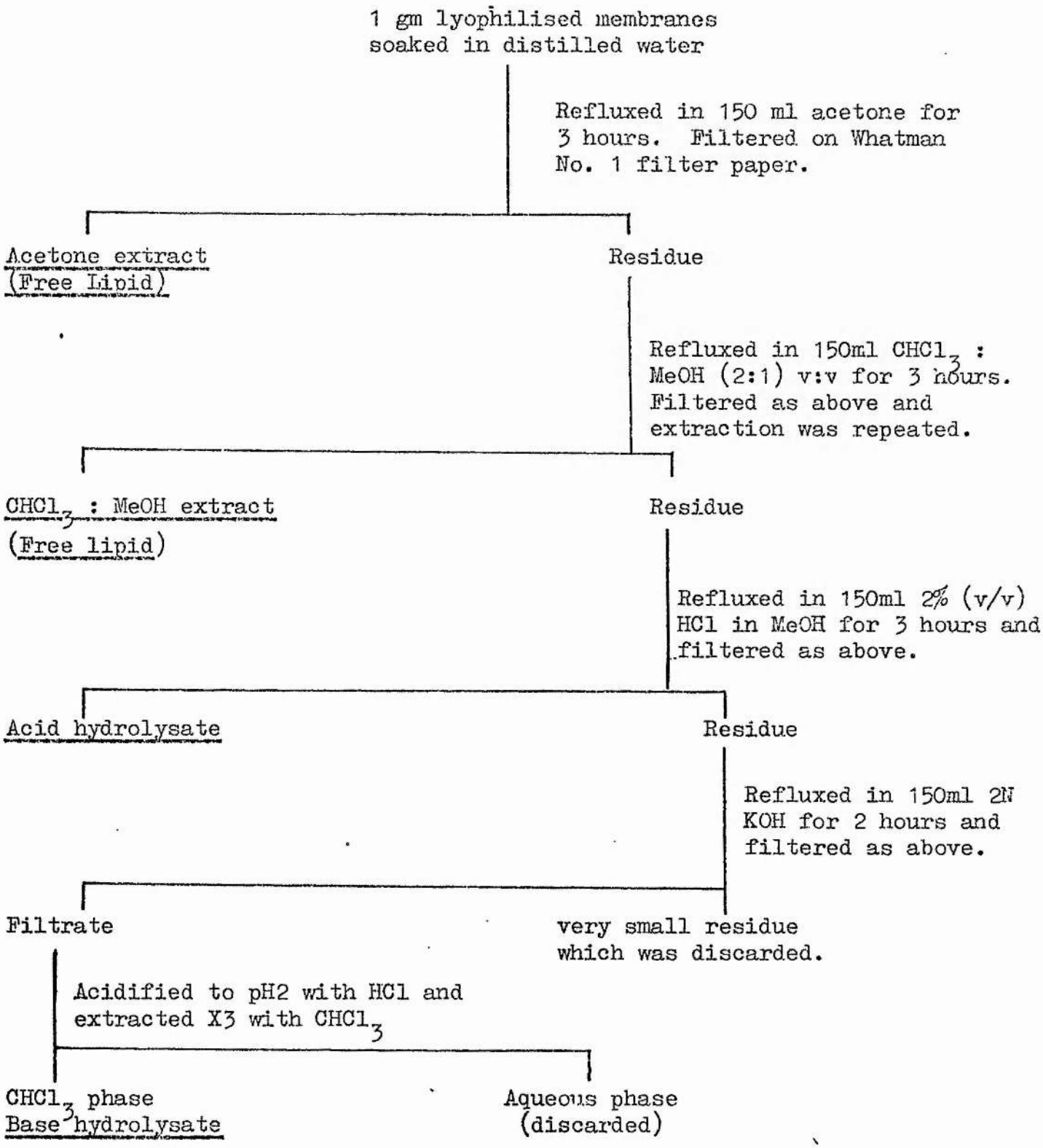
Purification

The extraction methods outlined above also extract non-lipid materials which can be removed by the following procedures:-

(1) Method of Folch, Lees and Sloane-Stanley (1957)

The lipid fractions were dried in vacuo and dissolved in $CHCl_3$: MeOH (2:1) v/v and partitioned with 0.05N NaCl, the ratio of organic solvent to 0.05N NaCl being 5:1 v/v. Non-lipid contaminants

FIG. M. IV LIPID EXTRACTION BY METHOD I



are removed in the aqueous layer. The main disadvantages to this method are:-

(a) Emulsions can form between the two phases which are hard to break.

(b) There is the possible loss of acidic, polar lipids into the aqueous layer.

(c) This method fails to remove inorganic phosphate.

(2) Method of Wells and Dittmer (1963)

This method has a lipid recovery of 98-100% and removes amino acids, carbohydrate, HCl, nucleotides and phosphate.

1gm washed and dried G_{25} fine sephadex was packed as a slurry in $CHCl_3$: MeOH : water (60:30:4.5) v:v:v into a 0.6 x 40 cm column. The lipid sample, dissolved in a maximum of 15 ml of $CHCl_3$: MeOH : water (60:30:4.5) v:v:v was added to the column and washed in with 5 ml $CHCl_3$: MeOH (2:1) v:v. The lipid was found in the effluent leaving the contaminants on the column. The non-lipid material held on the column can be eluted by removing the sephadex from the column and washing with methanol : water (1:1) v/v.

The former method is still very popular and is a faster process but the latter method is much more efficient. The washed lipid fractions were dried in vacuo and lyophilised to constant weight.

Separation of Lipid into Neutral Lipid, Glycolipid and Phospholipid Classes using Silicic Acid Column Chromatography

This fractionation was performed on the 'free' lipid extracted by Method 2 and purified by the method of Wells et al., (1963). The lipid was fractionated on a silicic acid column using the method or Vorbeck and Marinetti (1965) with the exception that in this case Mallinckrodt silicic acid was used. The best results were obtained when Mallinckrodt silicic acid (100 mesh) was activated for 12 hours at 110° and cooled in a vacuum desiccator prior to use.

10 gm silicic acid was packed as a slurry in 75 ml heptane into a column 1.75 cm in diameter to give a bed-height of 12cm. The column was washed with 60ml diethyl ether and 60 ml chloroform. The lipid sample containing 500-600 µgm phosphorus, dissolved in 5 ml chloroform, was applied to the column and washed in with two 3 ml portions of chloroform. The eluants were (applied) in the order:-

I	60 ml $CHCl_3$	- elutes neutral lipid
II	50 ml $CHCl_3$: acetone (50:50) v/v	elutes glycolipid
III	50 ml acetone	
IV	50 ml $CHCl_3$: MeOH (50:50) v/v	elutes phospholipid
V	50 ml MeOH	

In preliminary tests, the above fractions were monitored by analysis for phosphorus (method of Allen, 1940) and for carbohydrate (phenol-H_2SO_4 method). Each fraction was taken to dryness in vacuo and lyophilised to constant weight. The glycolipid fractions II and III were hydrolysed in 2M HCl for 3 hours at 105°. The hydrolysate was made acid free as before and the carbohydrate present identified

by GLC. Fractions I, II + III and IV + V (i.e. the neutral lipid, glycolipid and phospholipid fractions) were analysed for fatty acid composition. The free lipid, acid and base hydrolysates from the first lipid extraction method were also analysed for fatty acid composition.

Isolation of the Fatty Acids

Each fraction was dissolved in 100ml 10% (w/v) KOH/MeOH and saponified overnight at room temperature. The saponification mixture was diluted with two volumes of distilled water and extracted three times with diethyl ether to remove the non-saponifiable lipids. The aqueous phase was acidified with concentrated HCl to pH2 and extracted three times with diethyl ether. The combined ether extracts were washed with distilled water, dried over anhydrous Na_2SO_4 and the ether removed in vacuo.

Esterification of the fatty acids

The fatty acid residue was dissolved in 25 ml BF_3 : MeOH complex (BDH Ltd) and refluxed for 15 minutes. The reaction was terminated by the addition of two volumes of distilled water and the fatty acid esters were extracted into diethyl ether after the addition of 2 volumes distilled water (3 extracts). The combined ether extracts were washed with 1% (w/v) $NaHCO_3$ to remove traces of acid and finally with water. After drying over anhydrous Na_2SO_4, the ether extract was concentrated to a very small volume.

Hydrogenation of Fatty Acids

Fatty acid methyl esters were dissolved in 20 ml dry methanol and a few mgm of 10% palladium on charcoal catalyst were added. The mixture was shaken under an atmosphere of hydrogen for 30 minutes at room temperature. After filtration to remove the catalyst, two volumes of distilled water were added to the filtrate and the reduced fatty acid methyl esters were extracted into diethyl ether.

GLC of Fatty Acid Esters

A Pye model 104 Gas Chromatograph was used under the following conditions:-

5ft glass column (single) - 10% diethylene glycol succinate (DEGS) on 100-120 mesh celite or 5% Apiezon L (APL) on 100-120 mesh celite

		DEGS	APL	
Column temperature	:	190°	215°	isothermal
Detector oven temperature	:	210°	235°	
Injection point heater	:	220°	245°	
Attenuation	:	50×10^{3}		
Carrier gas (N_2) flow rate	:	45ml/min		
Air (to detector)	:	600ml/min		
H_2 (to detector)	:	45ml/min		
Chart speed	:	25mm/min	5mm/min	

The following fatty acids (BDH Ltd) were esterified as a mixture

and this standard mixture was injected before each sample was run:- n-octoic, n-decoic, lauric, myristic, palmitic and stearic acids ($C_8 \longrightarrow C_{18}$). For the standards, graphs were plotted of carbon number versus the logarithm of retention times (James plot). The peaks in the samples were identified after determining their equivalent chain lengths (ECL) from their retention times using the standard graph. Unsaturated fatty acids were identified by the disappearance of their peaks and a corresponding increase in the peaks of the equivalent saturated fatty acids, after reduction. Other peaks were characterised by comparison with data collected by Burchfield and Storrs (1962).

Peaks which were superimposed were resolved using an electronic curve analyser (Du Pont Ltd). The amount of each fatty acid component was determined from the individual peak areas which were calculated from the product of the peak height and retention distance. The relative amounts of each fatty acid was expressed as:-

$$\frac{\text{peak area}}{\text{total area of peaks}} \times 100\%$$

Extraction of the Carotenoid Pigments

Three methods were employed for extracting pigments from S. aurantiaca:-

I Ultrasonication (Method by Strang, 1968)

A suspension of bacteria in redistilled methanol (96%) was placed in a glass centrifuge tube, which was in turn immersed in an ice bath and subjected to ultrasonication at 60 watts output for a total of three-two minute periods (with one minute cooling intervals). Then the suspension in methanol was rapidly brought to the boil, subsequently cooled quickly and centrifuged at 2000g. The coloured supernatant was taken to dryness on a rotary evaporator and stored in the dark under nitrogen at $4^{o}C$. After three extractions, no more pigment was released, leaving the bacterial remnants virtually colourless.

II Vibrogen Cell Mill (Edmund Buhler)

The bacteria were suspended in redistilled methanol (96%) and 0.55mm (diameter) glass beads were added so that the ratio of bacteria: glass beads : methanol was 1:1:2 (v:v:v). The bacterial suspension was subjected to three-two minute periods of disintegration by vibrating glass beads interspersed by intermediary cooling periods of 1 minute. The suspension was continually cooled by running cold tap water through the outer jacket during the disintegration. Three extractions followed by centrifugation at 2000g left the remnants almost colourless. The three supernatants were combined, concentrated and stored as before.

III Chloroform : Methanol (2:1) v/v Extraction

The harvested bacteria were suspended in 0.1M Tris buffer (pH 7.5)

and digested overnight with lysozyme (Sigma Ltd) 150 µgm/ml. Then, the suspension was digested for 30 minutes with DNAase (30 µg/ml) which reduced its vicosity and the bacterial membranes were recovered by centrifugation at 35,000g. There was no loss of carotenoid into Tris buffer demonstrating the the pigments of S. aurantiaca are also located in the membrane. The removal of the outer cell wall facilitated a more efficient, and enabled a less vigorous, extraction of the carotenoid pigments.

The bacterial membranes were suspended in $CHCl_3$: MeOH (2:1) v/v and stirred on a magnetic stirrer for eight hours under an atmosphere of nitrogen, in the dark at 4^{o}. The suspension was centrifuged at 2,000g and the remnants extracted twice before they were colourless. The combined supernatants were concentrated and stirred as before. To ensure that all the pigment had been extracted by this method, the bacterial remnants were further extracted in methanol using the Vibrogen cell mill but no more pigment was released by this treatment. All solvents employed were redistilled before use.

Purification Procedures for Carotenoids

I Lipid Precipitation (Method of Blessin, 1962)

The total pigment in methanol was added to an equal volume of diethyl ether. A white flocculent precipitate of lipids (not carotenoids) was formed and centrifuged off.

II Saponification

Due to the presence of a high proportion of other lipids, saponification in 5% (w/v) methanolic KOH is a very useful purification step. However, some of the pigment fractions were found to be unstable to saponification, so this step was omitted for the total pigment and only attempted on individual pigment fractions.

Final purification of the total pigment

Resolution of the total pigment was first attempted using preparative thin layer chromatography (TLC). The only solvent system which gave a good separation using silica gel G (Merck) as adsorbent was benzene : methanol : acetic acid (87:11:2) v:v:v but artefacts appeared, presumably due to degradation in the presence of the acetic acid.

Column Chromatography

Various adsorbents for column chromatography were tried and the best pigment separation was obtained using Mallinckrodt silicic acid (100 mesh). Mallinckrodt silicic acid was activated at 110^{o} overnight and cooled in a vacuum desiccator. The glass column (1.5cm in diameter) was packed with silicic acid as a slurry in petroleum spirit (60-80^{o}) under gravity to give a final bed height of 30cm. A 2cm base of hyflo was used to increase the flow rate. The column was left overnight to equilibrate in petroleum spirit (60-80^{o}).

The total pigment extract (Method III), after an initial purification by the Blessin technique, was dissolved in 10ml petroleum spirit (60-80°) and applied to the column. A few mgm of quinol, (an antioxidant) were added to the pigment to help prevent oxidation and isomerisation of the pigment during column chromatography (Boot, 1959). The eluants in the order used were:-

I 15% diethyl ether in petroleum spirit (60-80°)

II 50% diethyl ether in petroleum spirit (60-80°)

III 50% methanol in petroleum spirit (60-80°)

The three crude fractions I, II and III were collected, concentrated and re-chromatographed using TLC.

Thin Layer Chromatography was employed with silica gel G (Merck) as adsorbent. TLC was also used on a preparative scale. The 20 x 20cm plates of silica gel G (Merck) (0.50mm thickness) were all pre-run with solvents prior to use to remove impurities present in the silica which would interfere with visible and UV absorption spectra of the pigments.

Fractions I, II and III were dissolved in diethyl ether and strip-loaded, using a Desaga applicator (Camlab. Glass Ltd), on the silica gel G (Merck) plates which had been activated at 110° for 30 minutes prior to use.

Fraction I

Using the solvent system Benzene : Petroleum spirit (60-80°) :

acetone (50:50:1) v:v:v, fraction I was resolved into two sub-fractions I_A and I_B. The purity of fractions I_A and I_B was checked by re-chromatography using the following solvents:-

I_A : Petroleum spirit (60-80°) : Diethyl ether (75:25) v:v

I_B : Benzene : ethyl acetate (3:1) v:v

Fraction II

This fraction formed a discrete band when chromatographed using the following solvents:-

Benzene : Petroleum spirit (60-80°) : acetone (50:50:6) v:v:v

Benzene : ethyl acetate (2:1) v:v

Fraction III

This fraction was resolved into two sub-fractions III_A and III_B using the solvent system 0.5% (v/v) pyridine in methanol. No other solvents would cause the migration of fraction III from the origin.

Characterisation of the Carotenoid Fractions

(1) Quantitative Determination (Method of Gillam and Stein, 1955)

The pigment fractions were dissolved in a known volume of petroleum spirit (60-80°) and the absorbances measured at the λ_{max} using a Unicam SP 600 spectrophotometer. The quantity of carotenoid present in solution was calculated using the following equation:-

$$W = \frac{D \times V \times 10}{E}$$

W = weight carotenoid in mgms

D = optical density of the solution at λ_{max}

V = volume of solution

E = optical density at the λ_{max} of a 1cm path length through a 1% (w/v) solution.

Since the S. aurantiaca pigments appear to belong to the α-carotene series, $E^{1\%}_{1cm}$ was taken as 2800 which is the value quoted for α-carotene (Schwieter, Bolliger, Chopard-Dit-Jean, Englert, Koflen, Koenig, Von Planta, Rueegg, Vetter, and Isler, 1965).

2. Absorption Spectra in the Visible and Ultraviolet (UV) Regions

The absorption spectrum in the visible and UV ranges was determined for each fraction in several different solvents (redistilled) using a Pye Unicam SP 800 spectrophotometer.

3. Infra red Spectroscopy (Unicam SP 200 I R Spectrophotometer)

The spectra of some fractions were determined in carbon tetrachloride and carbon disulphide in cells with NaCl windows and 1mm teflon spacers using a Unicam SP 200G instrument.

4. Partition Ratio

Petracek and Zechmeister (1956) showed that partition coefficients of carotenoids between the two phases of a 95% methanol-hexane system could be useful in structure determination because they demonstrated a relationship between functional group and partition behavior.

95% methanol was first equilibrated with hexane. Each carotenoid fraction was dissolved in a known volume of equilibrated 95% methanol

and shaken with an equal volume of equilibrated hexane. The optical density at λ_{max} was read in 95% methanol using a Unicam SP 600 spectrophotometer before and after partition with hexane.

5. Acetylation

Primary and secondary hydroxyl groups are readily acetylated by acetic anhydride in dry pyridine at room temperature, whilst tertiary hydroxyl groups do not react under these conditions. This technique was first developed by Kuhn and Sorensen (1938) and subsequently modified by Liaaen-Jensen (1962a).

Each fraction was dried in vacuo and dissolved in 1ml redistilled pyridine (dried over NaOH pellets). 0.1 - 0.2ml acetic anhydride (AR) was added and the reaction allowed to proceed at room temperature, in the dark, and under an atmosphere of nitrogen. The reaction was monitored by withdrawal of aliquots and TLC using 20 x 5cm plates, at regular time intervals. Silica gel G (Merck) adsorbent of 0.25mm thickness was used with the following solvents:-

Benzene : Petroleum spirit (60-86°) : Acetone (50:50: 0——→4) v:v:v,

the volume of acetone used depending on the polarity of the fraction being acetylated. Components were identified visually and under UV light and the R_f of each spot was measured. The number of components corresponds to the original compound, partially acetylated intermediate(s) and to the fully acetylated product. This method is only reliable for

three or less hydroxyl groups. The absorption spectra before and after acetylation were recorded to check if any degradation had occurred.

6. Trimethylsilylation (McCormick and Liaaen-Jensen, 1966)

This reaction is specific for tertiary hydroxyl groups. The acetylated product from (5) was dried in vacuo and redissolved in 0.5ml dry redistilled pyridine. 0.2ml hexamethyldisilazane and 0.1ml trimethylchlorosilane were added and the reaction was allowed to proceed for one hour at room temperature, in the dark and under an atmosphere of nitrogen. The reaction was terminated by the repeated additions of carbon tetrachloride (to remove excess silane) followed by concentration in vacuo. The product was dissolved in diethyl ether and examined by TLC as in (5). Under these conditions there should be a 95% conversion of a tertiary hydroxyl group to the TMS derivative.

7. Oxidation with Nickel Peroxide (Thirkell, Strang and Chapman, 1967)

This reaction is specific for hydroxyl groups allylic to the polyene chain and to isolated double bonds. The carotenoid fraction was dissolved in dry diethyl ether and nickel peroxide was added in the ratio 5mgm NiO_2 to 1mgm of material. The reaction was allowed to proceed at room temperature, in the dark and under an atmosphere of nitrogen. Samples were taken at intervals and any products were identified by TLC as before and by measurement of visible absorption spectra.

8. Dehydration by Acid Chloroform (Karrer and Leumann, 1951)

Allylic hydroxyl groups will react with HCl-chloroform resulting

in a decreased polarity and extension of the chromophoric system of the carotenoid.

The pigment was dissolved in 2ml $CHCl_3$ and 0.8ml 0.07N HCl : $CHCl_3$ was added. The reaction was followed by measurement of visible absorption spectra and by TLC as before. Under these conditions there should be a 71% elimination of an allylic hydroxyl group as water.

9. Methylation (Metcalfe and Schmidt, 1961)

This reaction is specific for carboxyl groups. The pigment was dried under vacuo and redissolved in 2ml dry redistilled methanol. 0.5ml BF_3 : MeOH complex (HDH, Ltd) was added and the reaction mixture was refluxed for 3 minutes. The reaction was terminated by the addition of two volumes of distilled water and the products removed into ether and examined by TLC as before.

10. Reduction with $LiAlH_4$ (Goodwin, 1956)

This test is specific for a carboxyl group. The pigment was dissolved in dry diethyl ether and a small quantity of $LiAlH_4$ added. The reaction was allowed to proceed at room temperature until there was no further evolution of gas. The yellow insoluble complex formed was decomposed by the addition of ethanol and the products were transferred into diethyl ether by the cautious addition of two volumes of water. The diethyl ether layer was washed with distilled water and dried over anhydrous Na_2SO_4 and the products were examined by

visible absorption spectra and TLC as before. The yield expected for this reaction is 75.80%.

11. Test for Epoxy and Aldehyde Groups (Karner et al, 1948)

A few drops concentrated HCl were added to an etherial solution of the pigments. The presence of an epoxy or aldehyde group results in the formation of a blue colour.

12. Test for Epoxide Groups (Yamamoto, Chichester and Nakaya, 1961)

This test is specific for epoxide and furanoid oxides. The dry pigment was mixed with dry powdered $HgCl_2$ in a 1:5 ratio (by weight). The mixture, in a sealed tube, was heated in a steam bath for one minute. The products were dissolved in acetone and examined by visible absorption spectra. Epoxides and furanoid oxides form intense blue and blue-green complexes with absorption maxima in the 600-700 nm region.

13. Iodine Isomerisation

This method is based on the observations of Zechmeister and Polgar (1943). The pigment was dissolved in acetone and 2 to 3 drops of iodine solution (0.1gm I_2 in 50 ml petroleum spirit) were added. The reaction mixture was exposed to weak sunlight for 3 hours, under an atmosphere of nitrogen. The intermediates and final products were examined by visible absorption spectra and TLC as before. The final equilibrium mixture contains predominantly the trans form, since this

is normally the most stable isomer (Weedon, 1971). Exceptions to this generalisation are some acetylenic carotenoids.

14. Saponification (Liaaen-Jensen, 1962b)

Only a portion of each fraction was saponified due to the instability of some of the pigments to saponification. The pigment was saponified in 5% (w/v) methanolic KOH, at room temperature, under an atmosphere of nitrogen, overnight. The products were removed into diethyl ether by the addition of two volumes of brine. The diethyl ether layer was washed to neutrality, dried over anhydrous Na_2SO_4 and concentrated in vacuo. The products were examined by TLC as before for the presence of esters.

Examination of fractions III_A and III_B

The high polarity of these fractions made them very difficult to work with. Acetylation was not possible before saponification probably because of the presence of large amounts of occluded lipids.

Saponification of III_A and III_B in 5% (w/v) methanolic KOH

During saponification about 50% of both III_A and III_B were lost into the aqueous layer. Some pigment was recovered into diethyl ether (III_{A1} and III_{B1}) and part formed an orange-red precipitate at the interphase (III_{A2} and III_{B2}). The precipitates III_{A2} and III_{B2} were then dissolved in methanol.

The following work was carried out on these fractions:-

(1) visible and UV absorption spectra were determined.

(2) Partition ratios were measured as before.

(3) Acetylation was attempted but III_{A1} and III_{B1} were degraded by acetic anhydride in pyridine and III_{A2} and III_{B2} were insoluble in pyridine.

(4) Spot tests.

(a) Spraying with 2% (w/v) ninhydrin in acetone to detects peptides and/or proteins.

(b) Spraying with 0.1M anisidine phthalate to detect reducing sugars.

(5) Carbohydrate Analysis

Fractions III_{A1}, III_{B1}, III_{A2} and III_{B2} were hydrolysed in 1M HCl for 4 hours at 105°. The hydrolysates were made acid free and analysed for sugars using GLC as before, and also spot tested with 0.1M anisidine phthalate spray.

Isolation and Characterisation of a Water-Soluble Complex from S. aurantiaca

Carotenoid pigments were extracted into 95% (v/v) methanol using the vibrogen cell mill. The three methanolic extracts, together with the bacterial remnants were saponified in 10% (w/v) methanolic KOH overnight, at room temperature, in the dark and under an atmosphere of nitrogen. After removal of the unsaponificable material in the usual manner, the aqueous phase was still pigmented. Subsequent dilution of the aqueous phase with five volumes of distilled water and

partitioning with ether caused the precipitation of an orange-red material at the interphase which was recovered, dried and dissolved in distilled water. This orange-red complex was first concentrated by placing it in dialysis sacking and blowing cold air over it.
The complex was further investigated by:-

(1) Tests for Homogeneity

(a) Disc gel Electrophoresis

This was performed on a sample of the concentrated material under identical conditions to those described previously.

(b) Sephadex G_{25} Gel filtration

After swelling G_{25} sephadex in 1% (w/v) NaCl solution overnight, the slurry was packed into a column to a bed of 25cm x 1.5cm. After equilibration, a concentrated solution of the sample in 1% (w/v) NaCl was applied to the column and elution performed with 1% (w/v) NaCl solution. The movement of the coloured material could be followed down the column and 5ml fractions were collected before and after the pigmented band was eluted. Each fraction was then tested for pigment on the Unicam SP 600 at the λ_{max} and for protein using ninhydrin and absorption at 275 and 215 nm.

(2) Solvent Extractions

The aqueous material was partitioned against diethyl ether, hexane and chloroform to see if any pigment could be easily removed.

(3) Absorption Spectra

This was read using an SP 800 spectrophotometer over the λrange 600--200 nm against a blank of distilled water.

(4) Spot Staining

The material was stained with 2% (w/v) ninhydrin in acetone to detect peptide or protein and with 0.1M anisidine phthalate to detect carbohydrate.

(5) Hydrolysis of the Complex

(a) The complex was made 1M with respect to HCl and hydrolysed at 105^{o} for 4 hours. The hydrolysate was made acid free as before and then spot tested for carbohydrate using 0.1M anisidine phthalate. The monosaccharide content of the hydrolysate was determined by GLC as before.

(b) The complex was made 6N with respect to HCl and hydrolysed at 120^{o} for 18 hours. The hydrolysate was made acid free and the amino acid composition determined using a Locarte autoanalyser.

(6) Determination of the S^{o}_{20} Value

The material was concentrated further in dialysis sacking as before. Assuming the initial concentration to be 100%, runs were done using concentrations of 100%, 75%, 50% and 25% on the Beckman Spinco model E analytical ultracentrifuge. Photographs of the sedimentation pattern were taken every 30 minutes for $2\frac{1}{2}$ hours after reaching the maximum speed of 59,780 rpm. The S values were plotted and the S^{o}_{20} value determined.

RESULTS

Morphology of S. aurantiaca

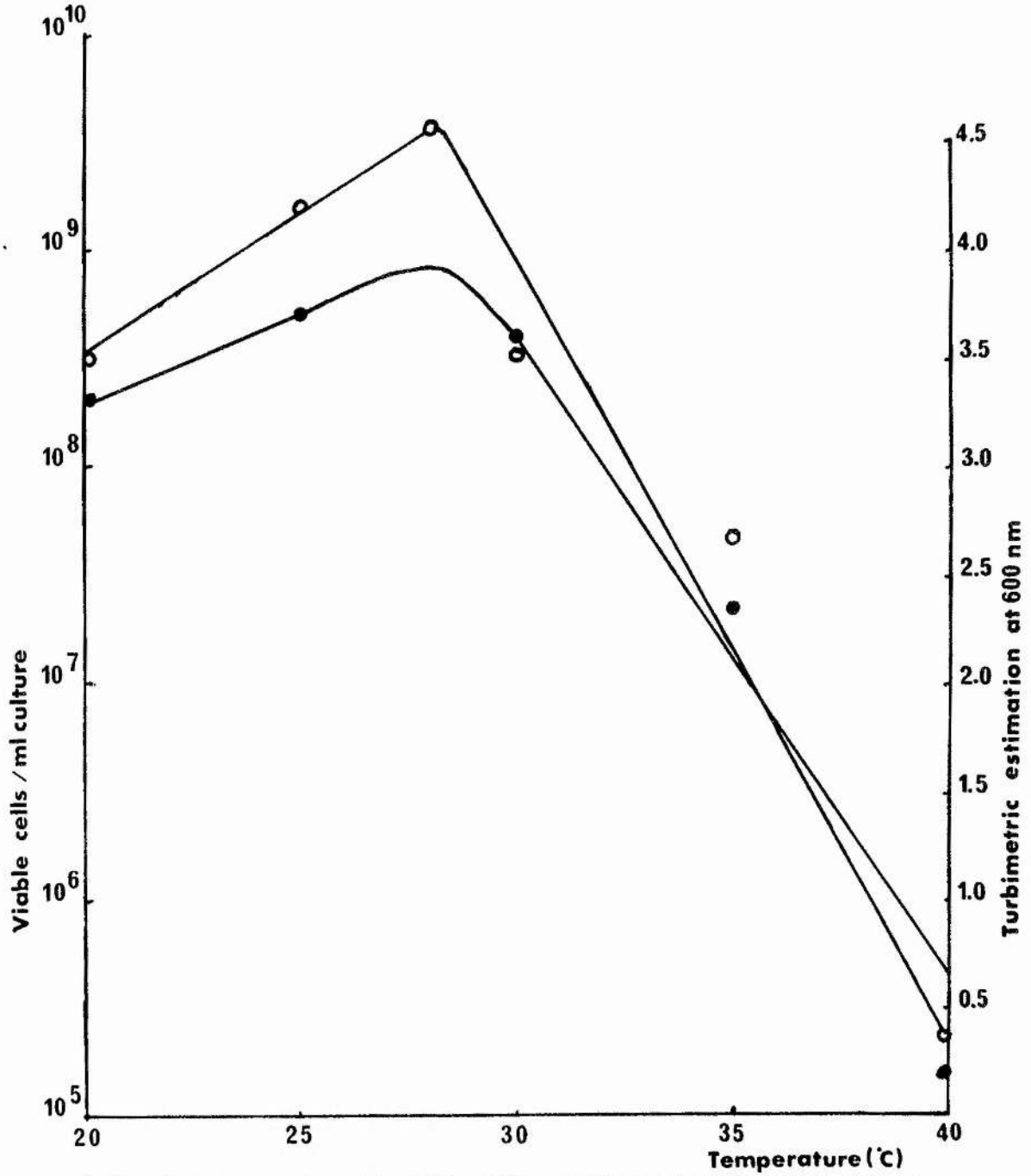
It was confirmed that S. aurantiaca (ATCC 146) is a Gram-positive coccus usually showing packet formation, although this was not always the case.

Investigation of optimum conditions for growth and pigmentation

Determination of the optimum temperature for growth.

The results for these experiments are presented in graph form. From figure R1, it is obvious that the optimum temperature for growth is 28^{o} and that comparatively little growth occurs above 40^{o}. Unfortunately, under the conditions of the experiment, it was not possible to grow S. aurantiaca below 20^{o}, since this was below room temperature and therefore the minimum temperature for growth was not determined.

The growth curves obtained using turbimetric readings at 600nm and by using the viable cell count technique were very similar, so only the viable cell count values are shown in figure RII. This similarity is unusual as the turbimetric method is normally less accurate than the viable cell count technique because the former method includes both viable cells and cell debris in suspension. From figure RII it can be seen that in liquid culture S. aurantiaca has a relatively short log phase at each temperature. This was followed by a period of rapid cell division resulting in maximum



1. Maximum number of viable cells reached at each temperature plotted against temperature ●—●

2. Maximum turbidity plotted against temperature ○—○

FIG. R.1 Growth of S. aurantiaca with temperature

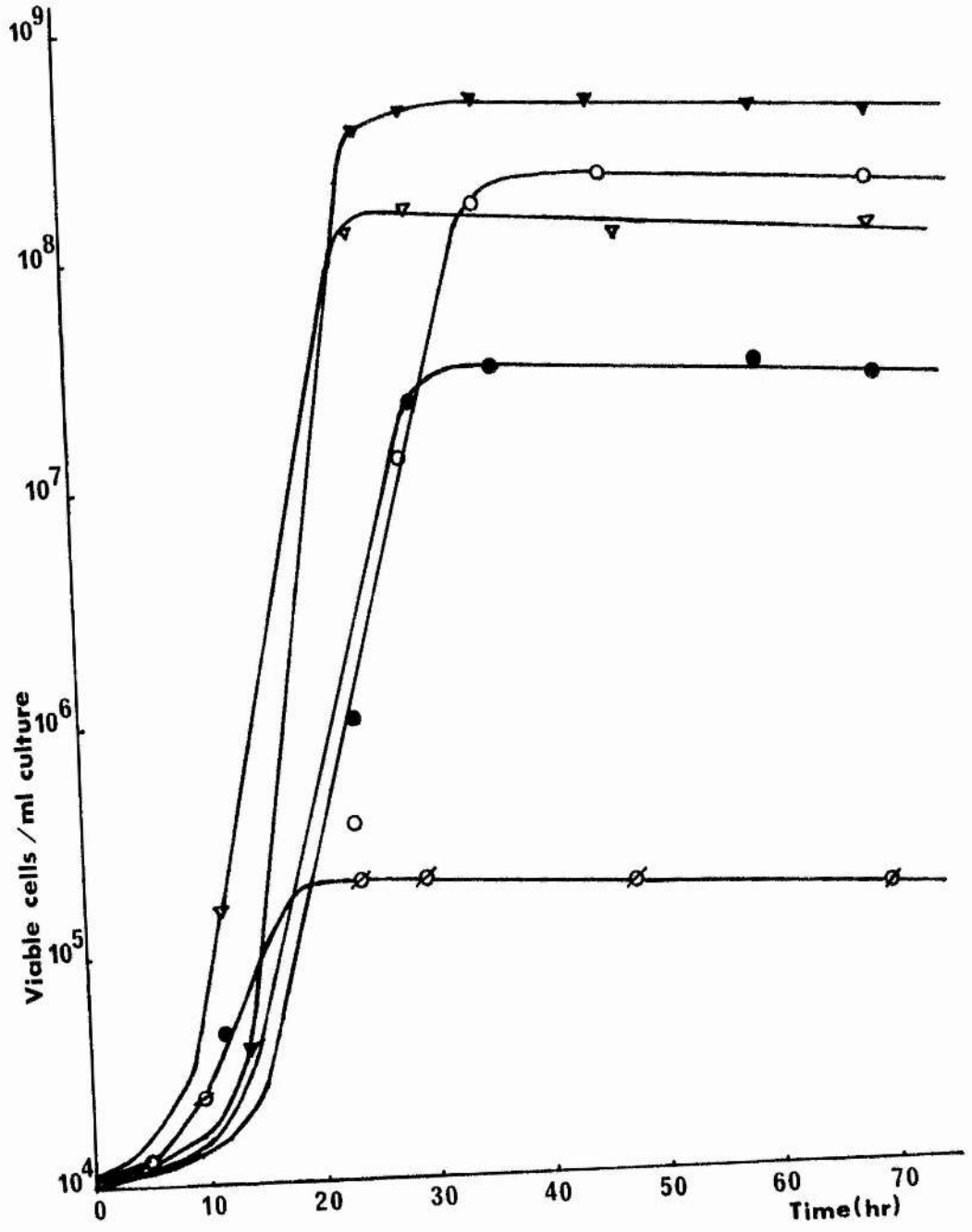


FIG. R [illegible] Growth of S. aurantiaca with time at various temperatures

- ○ 20°
- ▼ 25°
- ▽ 30°
- ● 35°
- ⌀ 40°

numbers after between 20 and 30 hours of culturing, dependant on the temperature, the only exception being the 20° culture. The stationary phase was maintained as a plateau up to 70 hours when readings were discontinued.

Determination of the Optimum pH for growth

Figure R. III shows that S. aurantiaca has a fairly narrow pH tolerance for growth, as growth only occurs to an appreciable extent between pH6.5 and 7.5. Growth is inhibited at pH5.5 and tolerated up to pH8.5, with the optimum pH slightly on the acid side of neutrality.

Determination of temperature and growth period for optimum pigmentation

Figure R. IV demonstrates that 28° is also the optimum temperature for pigmentation and that pigmentation is only maximal over a relatively short temperature range. It can be seen from figure R. V that, for each temperature, the peak in pigmentation is not reached until about 95 hours, that is, about 65 hours after peak numbers. Also, the peak in pigmentation is fairly short, the amount of pigment decreasing until about 140 hours when readings were discontinued. No pigmentation readings were obtained at 40°, as the yield of bacteria was too low for an accurate determination.

Chemical analysis of the cell membranes from S. aurantiaca

The approximate yield of the total membrane fraction per 100gm

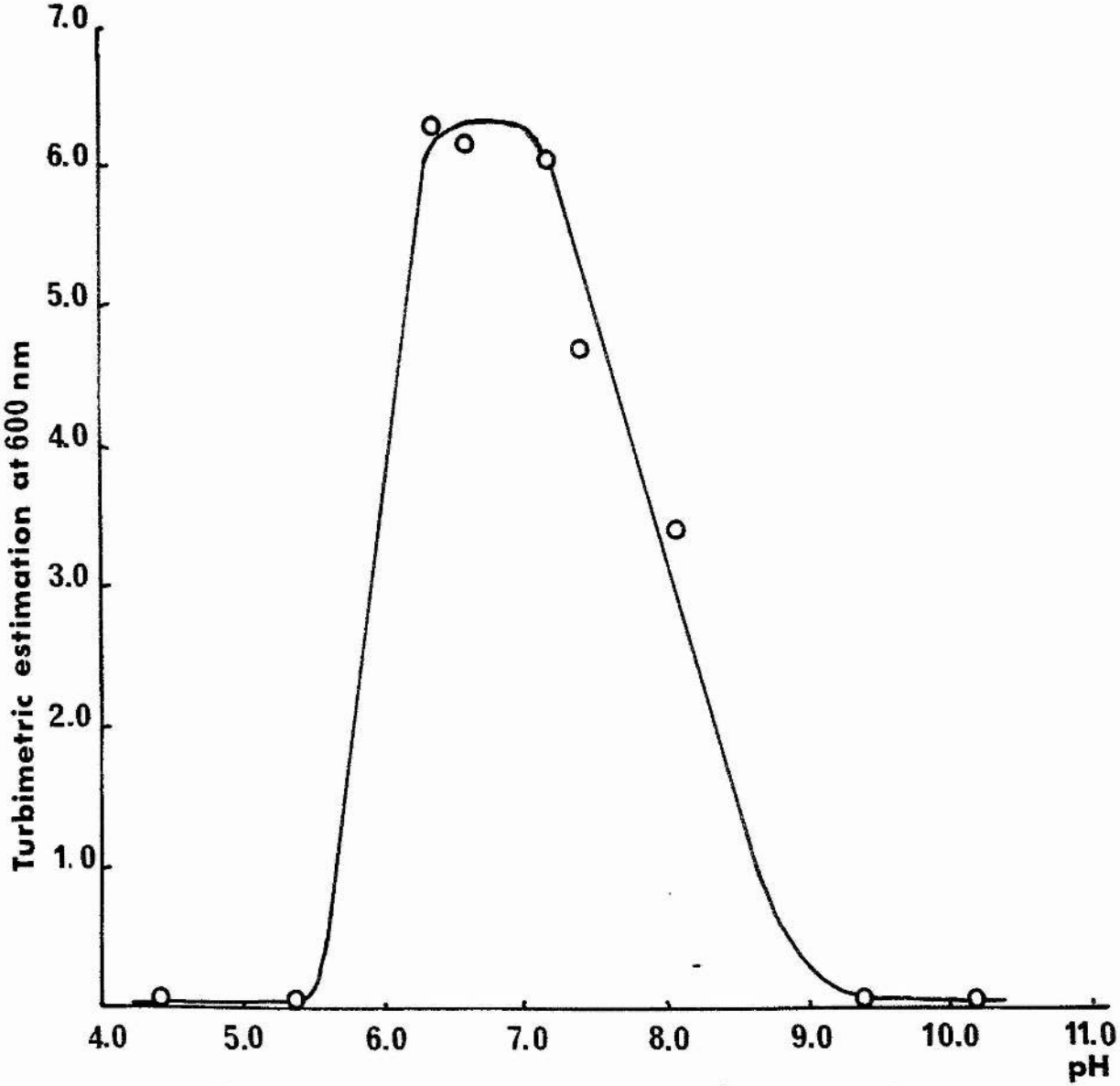


FIG. R. III Growth of S.aurantiaca at 28° with pH change

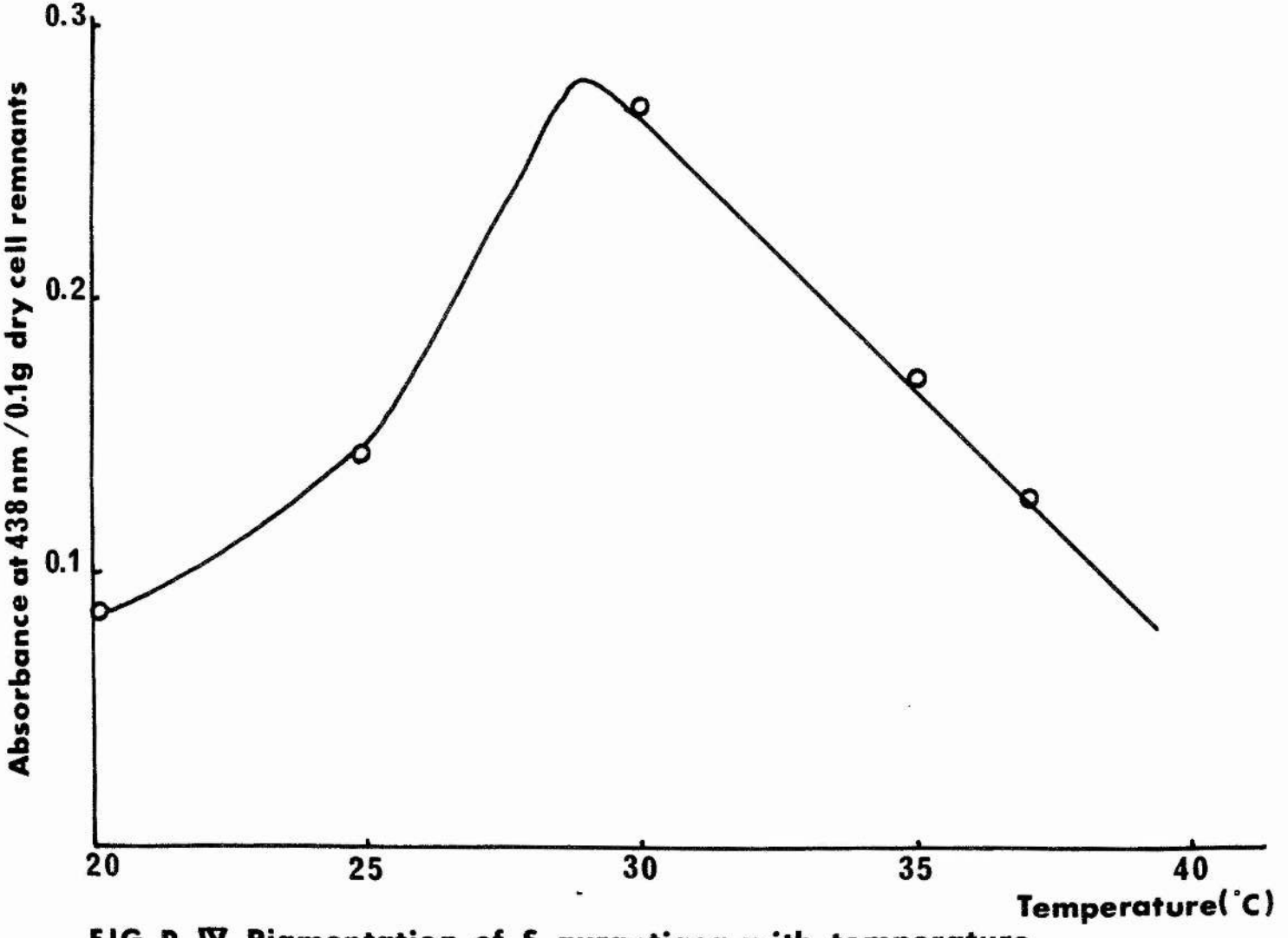


FIG. R. Ⅳ Pigmentation of S. aurantiaca with temperature

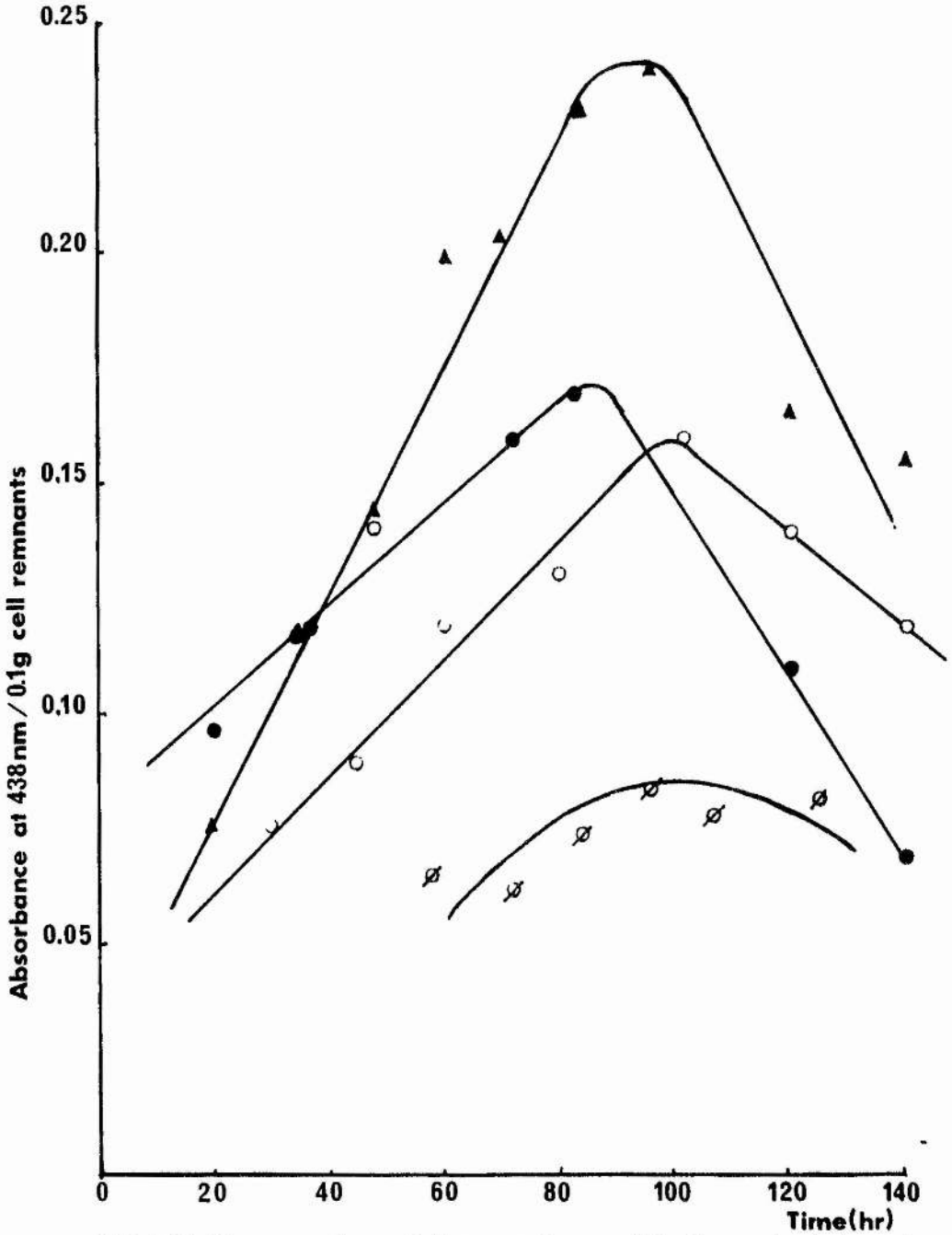


FIG. RV Pigmentation of S. aurantiaca with time at various temperatures

Ø 20°
○ 25°
▲ 30°
● 35°

wet wt. was 13.0gm (dry weight) for the 27 hour culture and 14.9gm (dry weight) for the 57 hour culture.

Moisture and Ash content

The results are shown in the table R.I and each is the average of three determinations.

Table R.I Percentage moisture and ash in the membrane preparations.

Age of membrane	Moisture	Ash
17 hours	5.4%	7.0%
27 hours	5.0%	1.8%
57 hours	4.9%	0:8%

The relatively high moisture content may be due to the inefficiency of the freeze-drier. There is a large decrease in ash content from the 17 hour membranes to the 27 hour and 57 hour membranes.

Determination of total phosphorus

The results are shown in table R.II and each is the average of six determinations:-

Table R.II Percentage phosphorus in the membrane preparations

Age of Membrane	Phosphorus
17 hours	0.560
27 hours	1.160
57 hours	1.015

It can be seen from these results that the phosphorus content doubles from the exponential to the stationary phase of growth.

Determination of RNA

The results are shown in Table R.III and each determination was performed in triplicate.

Table R.III Percentage RNA in the membrane preparations.

Age of Membrane	RNA (% of total organic material)
17 hours	0.38%
27 hours	1.49%
57 hours	1.99%

The difference in chemical composition between the 17 hour membranes and the 27 hour and 57 hour membranes is again illustrated by a much lower RNA content in the 17 hour membranes.

Protein Determination

The results are shown in Table R.IV and each is the average of six determinations by the method of Moore and Stein.

Table R.IV Percentage of protein in the membrane preparations.

Age of Membrane	Protein (% of total organic material)
17 hours	55.30%
27 hours	53.41%
57 hours	54.09%

There is comparatively little variation in the protein content with

age of culture. Although these figures should be reasonably accurate, it must be remembered that there may be other ninhydrin positive components in the test aliquots, albeit in low concentrations.

The results of the amino acid analysis are shown in table R.V where the amino acids are expressed as moles/1000 moles.

Table R. V Amino acid analysis of the membrane preparation.

	17 hour		27 hour		57 hour	
Amino Acid	M	Molar Ratio	M	Molar Ratio	M	Molar Ratio
Aspartic Acid	98.02	7.5	73.60	5.1	80.26	5.0
Threonine	56.03	4.3	45.50	3.2	46.90	2.9
Serine	47.00	3.6	38.70	2.7	39.31	2.5
Glutamic Acid	115.07	8.8	124.80	8.7	118.91	7.5
Proline	50.30	3.8	44.70	3.1	43.16	2.7
Glycine	95.01	7.3	85.10	6.0	84.56	5.3
Alanine	124.10	9.5	211.80	14.8	210.51	13.2
Valine	88.56	6.8	64.70	4.5	69.47	4.4
Methionine	4.44	0.3	14.30	1.0	19.71	1.2
Isoleucine	45.28	3.5	34.30	2.4	35.01	2.2
Leucine	90.28	6.9	70.80	5.0	76.08	4.8
Tyrosine	13.04	1.0	14.30	1.0	15.96	1.0
Phenylalanine	31.67	2.4	25.40	1.8	25.65	1.6
Lysine	44.86	3.6	77.20	5.4	61.33	3.8
Histidine	28.37	2.2	21.70	1.5	17.29	1.1
Arginine + Ornithine	65.77	5.0	52.50	3.7	55.49	3.5

From the above table, it can be seen that the predominant amino acids in all three membrane hydrolysates are alanine and glutamic acid.

There is also a relatively higher concentration of aspartic acid, glycine, valine and leucine. Thus, the apolar and acidic amino acids are present in much higher concentrations than the basic amino acids, and in turn their concentration is much higher than that of the aromatic and sulphur-containing amino acids. The major change in amino acid content is the large increase in the amount of alanine as the bacterial cell growth enters the stationary phase. With the exception of the fluctuation shown by lysine, the relative number of residues of all the other amino acids appear to show a small but significant decrease.

When the molar ratio of the amino acids in each of the preparations is examined, it is noticeable that none of the amino acids are present in whole number molar ratios. This is to be expected and is consistent with the view that in the membrane at any give time in the growth cycle, there must be several different proteins with diverse functions.

Extraction of Membrane Proteins

Method I (Maddy and Kelly, 1970)

After isolation of the fractions the following information was obtained:-

(a) High voltage Electrophoresis (HVE).

Since fraction 3 was only weakly ninhydrin positive and appeared to be mainly lipid, this fraction was not subjected to HVE. After running fraction 1 and 4 and staining with ninhydrin, only one band was seen and in each case the mobility of the bands was similar. Since these bands did not move very far from the origin, the proteins must

either be large or pH 4.75 is close to their isoelectric point or they are occluded in lipid in such a manner that their net charge is much reduced.

Unlike fraction 1 and 4, fraction 2 after running on HVE did not appear to stain with the ninhydrin at all. Since fraction 2 was initially ninhydrin-positive, its failure to stain after HVE may have been due to insufficient material being applied to the paper.

(b) Disc gel Electrophoresis

Again, fraction 3 was not applied to the system for the reason stated above. The migration distances observed for the bands in fraction 1,2 and 4 after different periods of time were as shown in table RVI where G.L = gel length and D.M = Distance migrated by amido-black positive material.

Table R VI Disc-gel electrophoretic pattern of protein fractions.

Fraction	1			2			4		
Run	1	2	3	1	2	3	1	2	3
G.L (mm)	57	60	60	57	60	60	57	60	60
D.M (mm)	38.0	15.0	11.5	44.0	20.0	14.5	38.5	16.0	11

In each case only one band stained with amido black and although the bands from fraction 1 and4 moved with similar mobility, that from fraction 2 had a significantly faster migration rate.

Since none of the fractions isolated was colourless, it is likely that all the fractions were in fact lipoproteins. Occlusion of protein in lipid may well explain why so few distinct protein-staining fractions have been resolved by either of the two techniques used.

Method 2 (Jeffrey, 1970)

An attempt was made to determine the number of protein components in F_A by the use of disc gel electrophoresis. Electrophoretic runs carried out for different time periods and then stained with amido black, always revealed only one band which had the same mobility as fraction 2 in Method 1.

It would seem that again a lipoprotein complex was being studied where the lipid interferes with the resolution of the protein components which must be present.

Carbohydrate Determination

Quantitative

The results are shown in table R VII and each is the mean of three determinations:-

Table R VII Percentage carbohydrate in the membrane preparations.

Age of Membrane	Total Carbohydrate (% of total organic material)
17 hours	7.19%
27 "	9.87%
57 "	11.10%

These values show an increase in total carbohydrate as the culture enters stationary phase.

Qualitative

Paper Chromatography

The $R_{glucose}$ values for the standards using the solvent system n-butanol : pyridine : water (6:4:3) v:v:v are shown in Table R VIII.

Table R VIII $R_{glucose}$ values of standard carbohydrates.

Monosaccharide	$R_{glucose}$
Arabinose	1.31
ribose	2.18
galactose	0.83
glucose	1.00
mannose	1.18

On staining with anisidine phthalate, the hexoses gave a brown spot and the pentoses a red spot.

The $R_{glucose}$ values for each component detected on the chromatograms in all three membrane hydrolysates are given in Table R IX.

Table R IX $R_{glucose}$ values of membrane carbohydrates.

$R_{glucose}$	Inference
2.15	ribose
1.16	mannose
0.99	glucose
0.83	galactose

Minor components with low $R_{glucose}$ values were also detected on the chromatograms and which could be amino sugars or glycopeptides.

GLC

The retention distances of standard sugars relative to α-methyl-D-glucoside (αM.D.G.) are shown in table R X.

Table R X $R_{\alpha M.D.G.}$ values for standard carbohydrates.

Monosaccharide	$R_{M.D.G.}$ α	β	γ
arabinose	0.340		
ribose	0.399	0.433	
rhamnose	0.359	0.490	
fucose	0.372	0.440	0.519
galactose	1.000	1.174	0.826
glucose	1.097	1.634	
mannose	0.787	1.180	
fructose	0.807	0.843	
xylose	0.504	0.629	
glucuronic acid	1.467	1.919	
arabitol	0.558		
ribitol	0.552		
mannitol	1.385		
sorbitol	1.422		
dulcitol	1.448		
glycerol	0.189	0.617	
n-acetyl glucosamine	2.261		
galactosamine hydrochloride	0.868		

N.B. α, β, γ denote the relative retention distances of the peaks corresponding to the appropriate anomer.

In all three membrane hydrolysates the presence of the following monosaccharides was suggested by their $R_{\alpha M.D.G.}$ values and their identity was confirmed by co-chromatography:- ribose, glucose, galactose and mannose.

Although, the absolute quantities of all the individual monosaccharides could not be calculated due to overlapping of some of the peaks, some conclusions could be drawn. For the 27 hour and 57 hour membrane hydrolysates, the relative proportions of the individual monosaccharides were approximately equal but differ from the 17 hour membrane hydrolysate.

The ratio of glucose to mannose in each case was calculated.

Table R XI Ratio of mannose to glucose in the membrane preparations.

Age of Membrane	α mannose : β glucose
17 hour	38.6 : 61.4
27 hour	74.9 : 25.1
57 hour	72.3 : 27.7

According to Sweeley et al, (1963) the ratio of α and β anomers for each sugar is a constant factor. From table R XI it can be seen that the 17 hour hydrolysate contains a higher concentration of glucose as compared with mannose, than the other hydrolysates but it was obvious from the GLC traces that the 17 hour hydrolysates had a

relatively lower concentration of galactose.

Figure R VI depicts a typical gas liquid chromatogram for the 57 hour membrane hydrolysate.

GLC of Hexosamines

Retention distances of the standard amino sugars relative to phenanthrene were calculated and are shown in table R XII.

Table R XII $R_{phenanthrene}$ values for standard amino sugars.

Amino sugar	$R_{phenanthrene}$	
	α	β
galactosamine hydrochloride	0.34	0.36
glucosamine hydrochloride	0.45	0.61

All three membrane hydrolysates contained only trace amounts of galactosamine and glucosamine, the values being too low for accurate determinations to be made. In most cases, the α galactosamine peak was missing, probably due to crystallisation of galactosamine prior to trimethylsilylation. The 17 hour hydrolysate contained approximately equal quantities of glucosamine and galactosamine whereas in the other hydrolysates glucosamine $>$ galactosamine.

A typical gas liquid chromatogram for the 27 hour membrane hydrolysate is depicted in figure R VII.

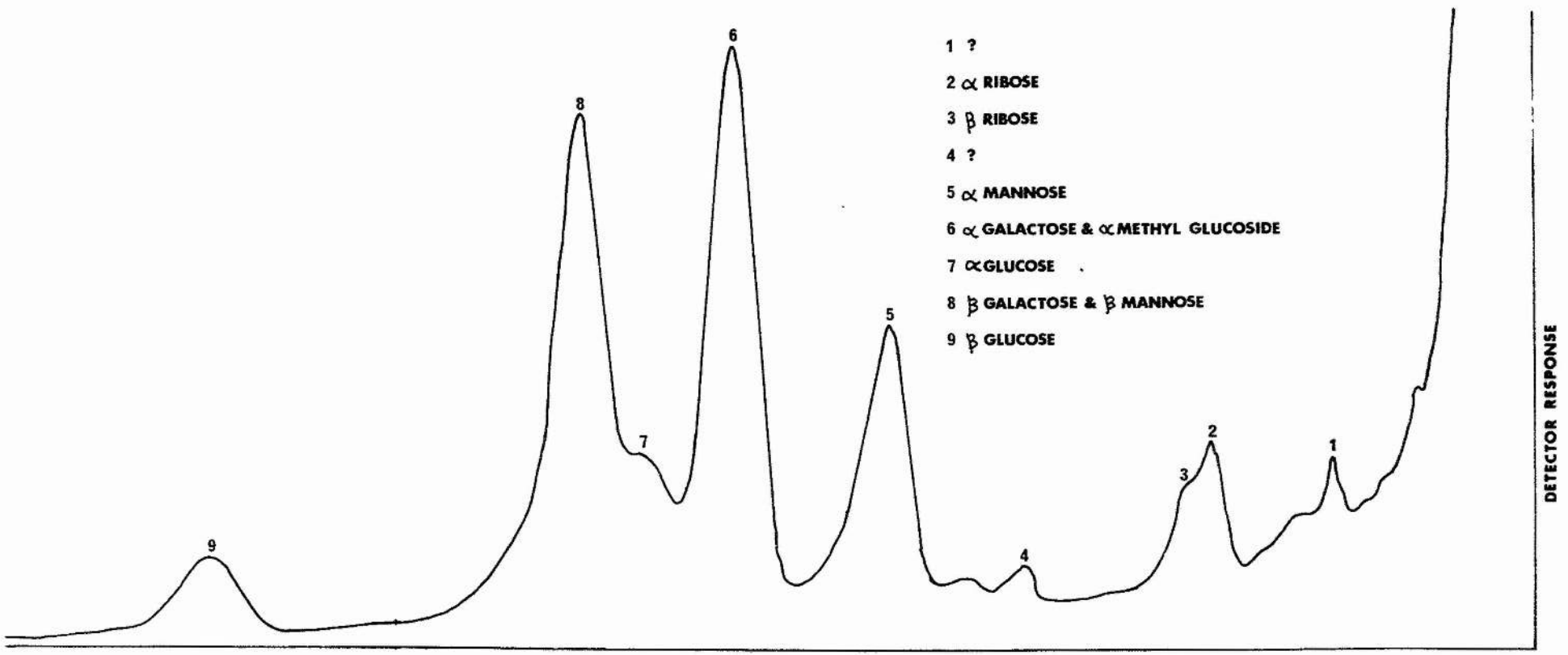


FIG. R VI G.L.C. OF T.M.S. DERIVATIVES OF CARBOHYDRATES FROM THE 57 HOUR MEMBRANES

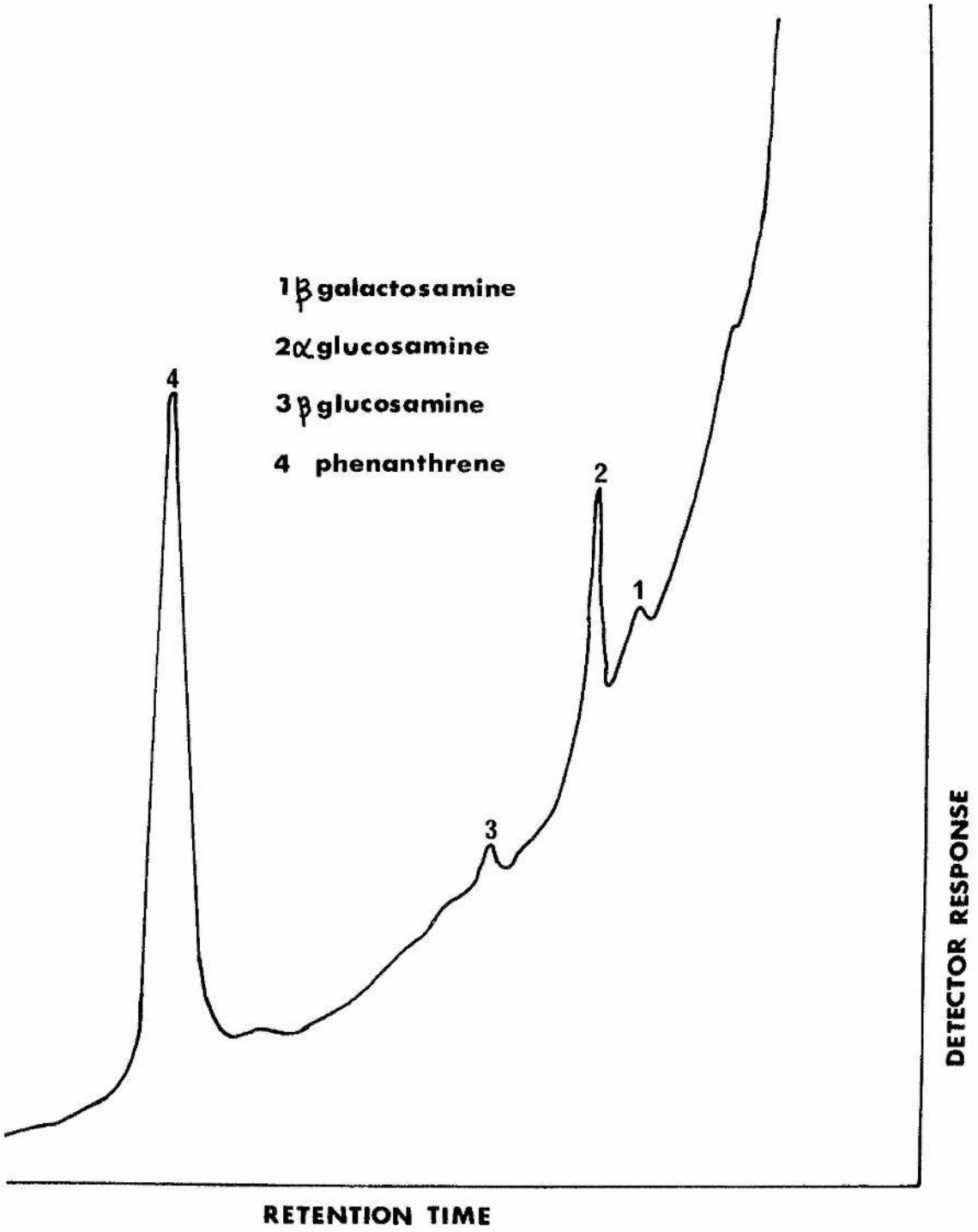


FIG. R. VII G.L.C. of hexosamines from the 27 hour membranes

Lipid Determination

Quantity

The results are shown in table R XIII as percentages of the total membrane organic material and each is the mean of three determinations. The figures in parenthesis denote the percentage of total lipid in each extract.

Table R XIII Total lipid content of the membrane preparations.

Method I (Huston et al, 1964)

Age of Membrane	Free lipid	Acid Hydrolysate	Base Hydrolysate	Total
17 hours	31.20 (92. 5%)	1.40 (4.15%)	1.11 (3.29%)	33.71
27 hours	9.27 (59.08%)	6.21 (39.57%)	0.21 (1.34%)	15.69
57 hours	5.14 (43.19%)	6.55 (55.04%)	0.21 (1.76%)	11.90

Method 2

Age of Membrane	Free lipid	Acid Hydrolysate	Base Hydrolysate	Total
17 hours	38.90 (98.00%)	0.71 (1.79%)	0.06 (0.21%)	39.67
27 hours	22.50 (94.10%)	1.23 (5.14%)	0.17 (0.71%)	23.90
57 hours	17.10 (88.20%)	2.12 (10.94%)	0.15 (0.77%)	19.37

The discrepancy between the two methods is partly due to the different methods of purification. Lipid extracted by Method I was washed by the method of Folch et al, (1957) whereas the lipid extracted by Method 2 was purified according to the process of Wells et al, (1963).

From the results of both extraction methods, the 17 hour membranes appear to have a much higher percentage of lipid than the other two membrane preparations.

Fractionation of the free lipid by silicic acid chromatography.

The results obtained for the free lipid extracts are shown in table R XIV.

Table R XIV Constitution of the free lipid of S. aurantiaca membranes.

Age of Membrane	Neutral Lipid (%)	Glycolipid (%)		Phospholipid (%
17 hours	73.54	Fraction II 11.65 Fraction III 5.04	16.69	9.76
27 hours	63.02	Fraction II 11.66 Fraction III 6.56	18.22	18.76
57 hours	62.09	Fraction II 4.58 Fraction III 7.84	12.42	25.49

All three preparations contain a very high percentage of neutral lipid. The percentage of phospholipid increases with age of the culture whereas the value for the other two fractions shows a compensatory decrease, although there is a fluctuation in the glycolipid content.

Examination of the glycolipid fractions for monosaccharides

The GLC results are shown in table R XV

Table R XV Monosaccharides of the membrane glycolipid.

Age of Membrane	% Total Glycolipid		Monosaccharides Detected
17 hours	Fraction II	: 69.8	Mannose: Galactose:: 39.6:60.4
	Fraction III	: 30.2	Mannose + trace (Glucose + Galactose)
27 hours	Fraction II	: 63.9	Mannose: Galactose : Glucose:: 66.9: 15.3 : 17.7
	Fraction III	: 36.1	Mannose : trace (Glucose + Galactose)
57 hours	Fraction II	: 39.87	Mannose : Galactose : Glucose :: 25: 48.1:26.9
	Fraction III	: 60.13	Mannose : trace (Glucose + Galactose)

As the monosaccharide content of fraction III is mainly mannose, and since as the cell ages the ratio of mannose to glucose + galactose in fraction II drops significantly, two points may be made. Firstly, table R XI shows that at 17 hours there is significantly more glucose than mannose and therefore most of the membrane glucose at that time is not incorporated into glycolipid. Secondly, the ratio of mannose to glucose in the membrane at 57 hours is almost 3:1. Since at 57 hours the carbohydrate in glycolipid fraction III is almost all mannose and this fraction accounts for 60% of the total glycolipid, it would appear that as the cell ages, much less glucose is incorporated into other membrane components than during the exponential phase.

GLC of the fatty acid methyl esters

The results for the GLC analysis of the fatty acids present in

the free lipid, the acid and the base hydrolysates (total lipid extracted by method I) are given in table R XVI and the fatty acids found in the neutral lipid, glycolipid and phospholipid (free lipid extracted by method 2) are shown in table R XVII. Fatty acids which constitute less than 1% of the total fatty acids are denoted as being present in trace amounts. A typical gas liquid chromatogram of the fatty acid methyl esters present in the acid hydrolysed lipid fraction from the 27 hour membranes, is depicted in figure R VIII.

Generally, fatty acids with carbon numbers ranging from C_{12} to C_{22} are found in significant quantities in the lipids of S. aurantiaca membranes and only trace amounts of fatty acids of carbon numbers ranging from C_9 to C_{12} are present. The majority of the lipid fractions have a saturated branched chain C_{15} fatty acid as the predominant fatty acid and also contain a relatively high proportion of other saturated, branched chain fatty acids. The exceptions are the neutral lipid fraction (Table XVII), and the free lipid and acid hydrolysed lipid fractions (Table XVI) from the 27 hour culture which all have a higher proportion of saturated, straight chain C_{16} and C_{18} fatty acids and the branched chain C_{19} fatty acids than the branched C_{15} fatty acid.

All the lipid fractions contain significant quantities of the straight chain, saturated C_{16} and C_{18} fatty acids and have a very

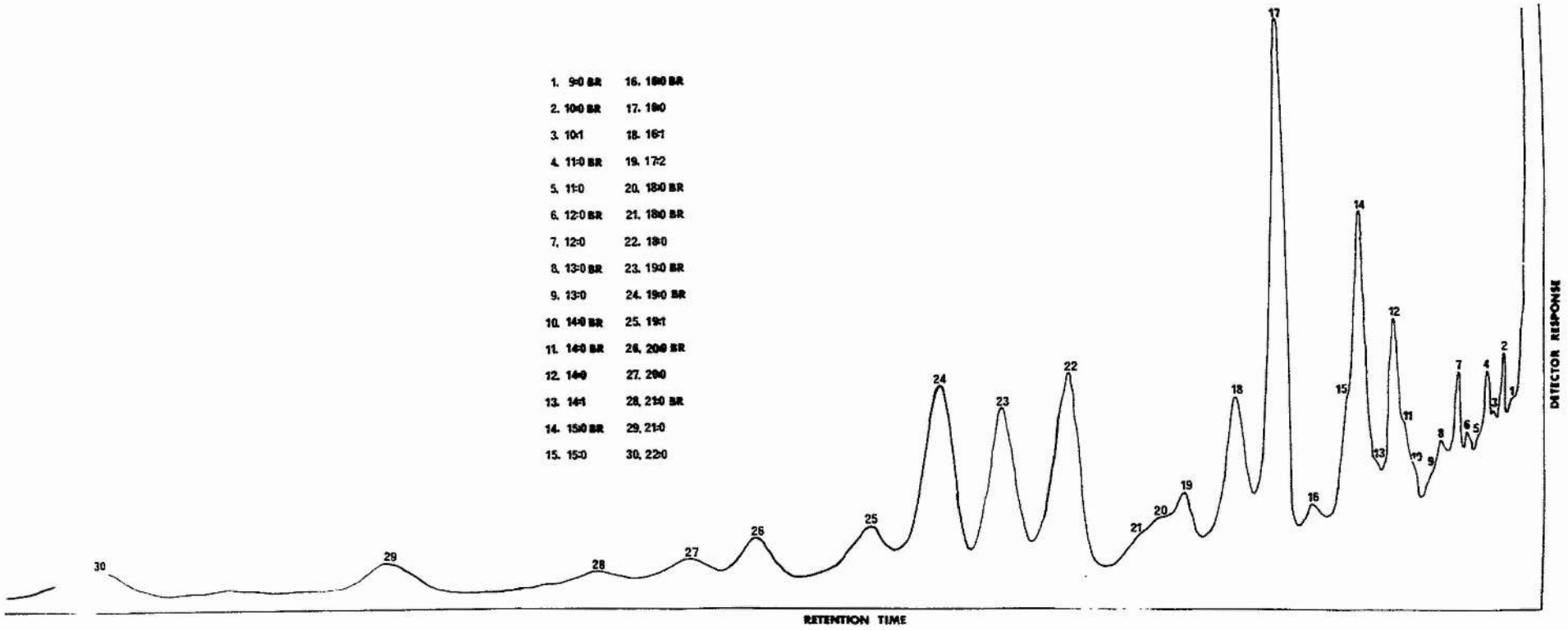


FIG. R VIII G.L.C. OF FATTY ACID METHYL ESTERS FROM THE ACID HYDROLYSED LIPID FRACTION OF THE 27 HOUR MEMBRANES

low proportion of unsaturated fatty acids. There are some fatty acid components which could not be identified by the methods used and are denoted in the tables by their ECL in parenthesis. These unidentified components could be hydroxy or cyclopropane fatty acids which are known to occur in bacteria (O'leary, 1962).

Table R XVI Fatty acid profiles of the total lipid isolates.

Fatty Acid	Free Lipid			Acid Hydrolysate			Base Hydrolysat		
.	17	27	57	17	27	57	17	27	57
BR 9:0	TR	TR	TR	TR	TR	TR	TR	TR	TR
9:0	-	TR	-	-	-	-	-	-	-
BR 10:0	TR	TR	TR	TR	TR	TR	TR	TR	TR
10:0	-	TR	TR	TR	-	TR	-	-	TR
10:1	TR	-	-	-	TR	-	TR	TR	-
BR 11:0	TR	1.01	TR	TR	TR	TR	TR	TR	TR
11:0	-	TR	TR	-	TR	-	-	-	-
BR 12:0	TR	TR	TR	-	TR	TR	TR	TR	TR
12:0	TR	1.37	1.15	2.54	1.14	TR	TR	1.02	TR
BR 13:0	-	TR	-	-	-	-	-	-	TR
BR 13:0	8.34	1.53	4.01	2.15	TR	1.02	.92	TR	TR
13:0	-	-	-	-	TR	-	-	TR	TR
BR 14:0	4.44	1.53	8.01	TR	TR	4.27	-	2.01	TR
BR 14:0	-	-	-	-	TR	-	-	-	2.5
14:0	3.07	4.30	8.68	4.55	2.48	5.44	2.74	3.37	3.4
14:1	--	TR	-	-	TR	-	TR	TR	TR
BR 15:0	65.78	7.78	24.99	22.07	5.70	46.11	6.70	17.08	11.8
15:0	-	1.27	-	-	2.38	-	4.52	-	2.0
BR 16:0	TR	1.07	3.17	1.61	1.10	1.26	TR	1.28	1.1
16:0	3.05	15.91	12.33	14.35	15.28	19.33	10.59	15.97	16.6

continued overleaf.

Table R XVI continued

Fatty acid	Free Lipid			Acid Hydrolysate			Base Hydrolysate		
	17	27	57	17	27	57	17	27	57
16:1	TR	3.87	2.48	4.55	5.28	1.50	2.53	4.15	2.38
BR 17:0	.98	.98	2.78	TR	-	1.14	TR	TR	TR
(17.2)	TR	2.87	1.28	1.77	2.78	TR	9.28	1.90	3.12
BR 18:0	TR	1.19	1.31	TR	1.60	TR	1.02	1.84	1.10
BR 18:0	-	-	1.63	-	1.05	-	-	TR	1.77
18:0	1.73	9.66	11.54	11.63	10.15	9.96	7.87	15.22	13.06
(18:3)	-	1.75	-	-	-	-	-	-	2.23
BR 19:0	2.22	8.00	4.52	11.46	9.80	2.91	11.66	6.28	5.58
BR 19:0	1.06	8.80	2.11	2.40	12.66	1.31	-	.98	TR
19:1	TR	2.00	2.09	2.94	4.28	2.13	1.58	2.14	1.76
BR 20:0	TR	2.39	-	1.76	-	-	10.42	1.58	2.56
BR 20:0	TR	3.88	1.63	3.92	4.56	TR	3.17	3.03	2.96
20:0	1.97	3.43	2.41	1.96	3.40	TR	2.23	3.17	1.86
BR 21:0	-	-	TR	-	2.71	-	1.11	-	2.80
BR 21:0	-	2.95	TR	6.31	-	-	10.10	1.16	4.86
21:0	2.63	2.30	TR	-	4.66	-	1.78	5.70	-
BR 22:0	-	1.54	-	-	-	-	-	-	-
22:0	-	5.30	TR	-	5.00	-	7.83	7.39	8.99

Table R XVII Fatty acid profiles of the neutral lipid, glycolipid and phospholipid fractions.

Fatty Acid	Neutral Lipid			Glycolipid			Phospholipid		
	17hr.	27hr.	57hr.	17hr.	27hr.	57hr.	17hr.	27hr.	57hr
BR 9:0	TR	-	TR	1.92	-	TR	TR	-	TR
9:0	-	-	-	-	-	TR	-	-	-
BR 10:0	TR	TR	TR	TR	TR	TR	TR	TR	-
10:0	-	-	-	-	-	TR	-	-	TR
BR 11:0	TR	-	TR	-	TR	-	-	TR	-
11:0	TR	TR	-	TR	-	TR	TR	-	TR

continued overleaf

Table R XVII continued.

Fatty Acid	Neutral Lipid			Glycolipid			Phospholipid		
	17hr.	27hr.	57hr.	17hr.	27hr.	57hr.	17hr.	27hr.	57hr.
BR 12:0	-	TR	TR	-	TR	TR	-	TR	TR
12:0	TR	1.64	TR	.95	1.50	2.26	TR	1.50	1.19
BR 13:0	-	1.48	-	-	-	-	-	-	-
BR 13:0	4.80	2.66	3.05	4.24	3.71	3.83	5.12	3.71	6.96
BR 14:0	2.33	2.84	6.13	3.17	6.18	8.25	3.60	6.18	10.15
14:0	2.64	8.43	7.80	3.38	9.12	8.44	3.53	9.12	9.96
14:1	-	-	-	-	-	4.44	-	-	-
BR 15:0	37.81	7.98	15.83	60.99	32.41	32.37	69.16	32.41	40.44
15:0	-	3.00	-	-	4.89	-	-	4.89	-
BR 16:0	TR	1.77	1.82	TR	3.30	2.18	TR	3.30	1.89
16:0	5.16	8.26	10.62	3.52	7.09	8.45	3.56	7.09	8.86
16:1	1.57	6.17	2.90	1.72	6.46	3.24	1.16	6.46	3.50
BR 17:0	2.57	1.33	3.73	TR	-	1.07	TR	-	-
(17:2)	1.39	TR	1.13	TR	1.44	2.54	TR	1.44	2.20
BR 18:0	TR	2.65	TR	TR	2.34	-	1.66	2.34	TR
18:0	19.01	14.59	21.79	4.23	2.97	4.40	2.18	2.97	2.71
BR 19:0	4.81	6.23	3.44	4.25	4.28	4.20	2.19	4.28	3.14
BR 19:0	5.56	15.02	8.81	1.74	-	2.99	TR	-	2.62
19:1	2.90	3.69	2.99	1.24	2.31	2.87	1.15	2.31	1.69
BR 20:0	1.56	3.76	2.85	1.04	5.58	5.03	1.02	5.58	2.47
20:0	-	3.36	1.00	1.46	5.15	TR	1.07	5.15	-
BR 21:0	-	-	-	1.46	-	-	-	-	-
BR 21:0	5.44	3.37	4.89	1.58	7.73	1.04	TR	7.73	-

A summary of the overall chemical composition for the 17 hour, 27 hour and 57 hour membranes is given in table R XVIII.

Table R XVIII Quantitative chemical composition of the membranes.

Age of Membrane	Moisture	Ash	Phosphorus	Carbohydrate*	Lipid*	Protein*	RN
17 hour	5.4	7.0	0.56	7.19	39.67	55.30	0.
27 hour	5.0	1.8	1.160	9.87	23.90	53.40	1.
57 hour	4.9	0.8	1.015	11.10	19.37	54.10	1.

* Expressed as the percentage of the total organic material

Age of Membrane	Total Organic Material
17 hour	102.54
27 hour	88.66
57 hour	86.56

The drop in the figures for recovery of organic material may be due to increasing difficulty in extracting the total lipid from the membranes of cells from older cultures.

Extraction of the Carotenoid Pigments from S. aurantiaca

Methods I and II which involved the extraction of carotenoids by either ultrasonication or by the use of vibrating glass beads, ensured a virtually total extraction of thepigments since at the end of the extraction procedure, the bacterial remnants were almost colourless. Both methods were, however, discontinued since subsequent work with the pigment extracts particularly spectral data obtained, revealed that a considerable amount

of pigment degradation occurred during isolation. This degradation could be brought about by local heat production even though the extraction vessels were kept cool either by the use of an ice bath (Method I) or by circulating cold water around the stainless steel extraction thimble (Method II).

The third extraction method involving the use of chloroform: methanol (2:1) v:v was selected for pigment extraction since a total pigment recovery from the membranes was achieved without associated degradation and artefact production. However, one major disadvantage of this method is that it also extracts a high proportion of other membrane lipids which made initial purification of the pigment extract imperative.

Purification procedures employed to remove other extracted material

Although the lipid precipitation method of Blessin did not cause pigment degradation as indicated by visible absorption spectra, the method did not remove all of the other lipids from the extract, but these could be removed from the free pigment fractions by column chromatography and TLC. Whereas saponification is normally employed for further carotenoid purification, this technique could not be used in this case as some of the pigments from S. aurantiaca membranes were found to be very unstable to alkaline hydrolysis. This process was therefore only attempted on aliquots of pigment fractions in

order to determine the presence or absence of esters.

The final purification of the pigment fractions from the silicic acid column was achieved by T.L.C. using silica gel G (Merck) (0.55mm) with the solvents given in table R XIX. The R_f values of the various pigment fractions in the different solvent systems are also shown.

Table R XIX T.L.C. separation of the pigment extract.

Fraction		Solvent System	R_f
I_A	(a)	Benzene : Light petroleum (b.p.60-80°): Acetone :: 50:50:1 :: v:v:v	0.93
	(b)	Light petroleum (b.p.60-80°): Ether :: 75:25 :: v:v	0.84
I_B	(a)	Benzene : Light petroleum (b.p.60-80°): Acetone :: 50:50:1 :: v:v:v	0.32
	(b)	Benzene : Ethyl acetate :: 3:1 :: v:v	0.75
II	(a)	Benzene : Ethyl acetate :: 2:1 :: v:v	0.72
	(b)	Benzene : Light Petroleum (b.p.60-80°): Acetone :: 50:50:6 :: v:v:v	0.45
III_A	(a)	0.5% (v/v) Pyridine in methanol	0.87
III_B	(a)	0.5% (v/v) Pyridine in methanol	0.00

The results of the various tests carried out on fraction I_A, I_B and II are shown in table R XX and typical absorption spectra recorded for these three fractions are seen in Figure R IX.

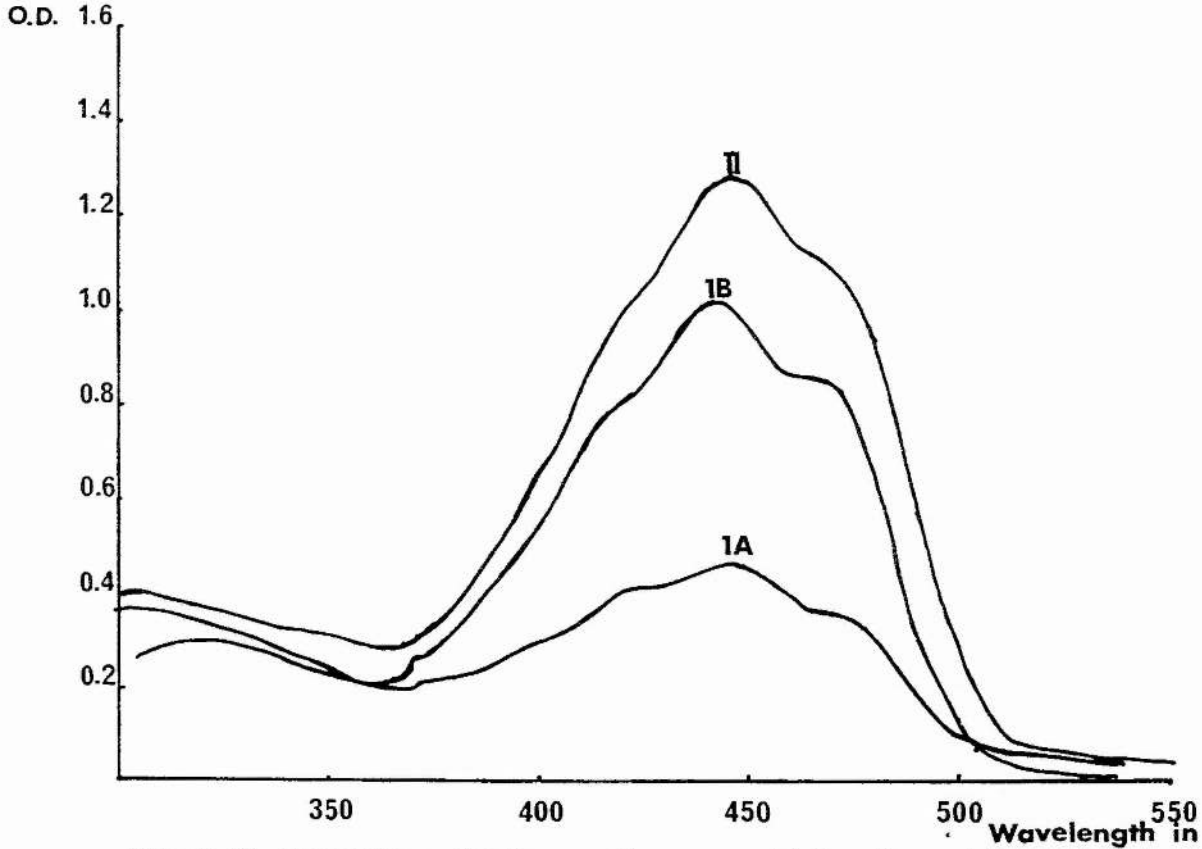


FIG. R. IX Absorption spectrum of carotenoid fractions 1A in petroleum spirit(bp.60-80)
1B & II in hexane

Table RXX The results of chemical tests on fraction I_A, I_B, and II.

Test	Fraction		
	I_A	I_B	II
% Total Pigment	0.61	4.05	23.93
Absorption Spectra-λ_{max} in:-			
(a) Light Petroleum (b.p.60-80°)	420,446,475	420,443,472	430,447,475
(b) Hexane	No data	420,443,470	421,445,474
(c) Ethanol	425,447,475	No data	422,446,475
(d) Acetone	424,450,478	No data	No data
(e) Carbon Disulphide	450,474,505	450,473,505	(448),475,505
U.V. region	No cis peak	No cis peak	No cis peak
Partition ratio (95 : MeOH : Hexane)	0 : 100	24 : 76	89 : 11
Acetylation	Negative	Maxiumum No. of spots detected = 2 (original + one ester) ie one Primary or one Secondary - OH group	Maximum No. of spots detected = 3 (original + 2 esters) i.e. two primary or secondary - OH groups.
Oxidation with nickel peroxide	Negative	Negative ie -OH not allylic	Pigment unstable to test
HCl/$CHCl_3$ Dehydration	Negative	Negative	Negative - OH groups not allylic
Trimethylsilylation of esters	Negative ie no tertiary - OH groups	Negative no tertiary - OH group	Negative - no tertiary - OH group.

Table R XX continued

Test	Fraction		
	I_A	I_B	II
Reduction of esters with $LiAlH_4$	Negative - no other reducible group.	Negative - no other reducible group.	Negative - no other reducible group
Aldehyde group test	Negative	Negative	Negative
Epoxy group test	Negative	Negative	Negative
Methylation	Negative - no COOH	Negative - no COOH	Negative - no COOH
Saponification	Stable, No change i.e. No ester present	Stable, no change - no ester present	Unstable but gives unchanged material and degraded artefacts - No ester present.
Iodine Isomerisation	No data	Single isomer produced, reverting to all-trans after 24 hours	Single isomer produced, reverting to all-trans after 24 hours.
Tentative identification	α-carotene	Zeinoxanthin	Dihydroxy α-carotene NOT lutein

From table R XX the following comments may be made regarding these fractions.

Fraction I_A

The absence of any oxygen function as shown by the tests is in accordance with the observed partition ratio. That I_A is not an ester was shown since the saponification product co-chromatographed with the

original material. The λ_{max} in the visible region suggests a chromophore of ten conjugated double bonds and also suggests that I_A is α-carotene, as shown by co-chromatography with authentic α-carotene in several solvent systems. Furthermore, the all-trans nature is shown by the absence of a cis peak at 260-300nm in the U.V. region. During the final purification of I_A, less polar, fluorescent (presumably hydrocarbon) components were also detected. They were not present in sufficient concentration to permit isolation and identification but they were probably hydrocarbon precursers of the Porter-Lincoln series of compounds. No other coloured carotenes were detected.

Fraction I_B

The partition ratio and the results of the chemical tests are indicative of a monohydroxy carotenol isolated as a free (not esterified) all-trans pigment. The λ_{max} in the visible region again showed the presence of a chromophore of ten conjugated double bonds and suggested that the carotenol was derived from α-carotene. However, the results of oxidation with nickel peroxide and dehydration with $HCl/CHCl_3$ showed that the hydroxyl substitution was not allylic to the polyene chain. This finding, along with a comparison of the λ_{max} with values quoted in the literature, would suggest that fraction I_B is zeinoxanthin (3-hydroxy-α-carotene) and not α-cryptoxanthin

(3'-hydroxyl-α-carotene) since the latter has an allylic substitution.

Fraction II

The partition ratio and the results of the chemical tests are indicative of a dihydroxy carotenol. The absence of a cis absorption peak at 260-300nm and the result of the iodine isomerisation test showed that the compound was extracted in the all-trans form.

It was found that fraction II was unstable to saponification and when the products of this procedure were recovered and chromatographed with the original fraction by TLC on silica gel G (Merck) (0.50mm with the solvents benzene : light petroleum (b.p. 60-80°) : acetone (50:50:5) v:v:v, one fraction (approximately 35% of the total) was similar to the original material. This was confirmed by the absorption spectrum of the fraction. The other three fractions resolved from the saponification etherial extract were shown to be artefacts with substantial alteration to their chromophore. On the assumption that the one saponification product which co-chromatographed with the original material was the only undergraded fraction, it can be deduced that fraction II is a free pigment and not an ester.

Since neither of the two hydroxyl substitutions (shown by acetylation) appeared to be allylic to the polyene chain, and on consideration of the λ_{max}, it is probable that this fraction is a dihydroxy α-carotene but not lutein. The latter (3,3'-dihydroxy-α-carotene) is stable to

oxidation with nickel peroxide and has one allylic hydroxyl substitution.

The chemical tests showed the absence of carboxyl, tertiary hydroxyl or keto groups in any of these fractions. Some of the degradation products produced by saponification of fraction II were also tested for the presence of epoxy or furanoid groups to show that the altered chromophores were in fact artefacts and not due to epoxides.

Fractions III_A and III_B

The original fraction III which constituted 71.41% of the total pigments was resolved, as described in the "Methods" section into two sub-fractions, III_A and III_B, which respectively constituted 35.41% and 36.00% of the original pigments. Absorption maxima recorded for these sub-fractions in four different solvents are shown in Table R XXI and figure R X shows their absorption in the visible region of the spectrum.

Table R XXI Absorption maxima of fraction III_A and III_B

Solvent	III_A	III_B
Light Petroleum (b.p. 60-80°)	431 454 ?	- 446 -
Diethyl Ether	428 454 482	428 454 480
Methanol	428 450 471	430 447 470
Carbon disulphide	(450) 475 (500)	(450) 473 502
U.V. absorption maxima	230 273 294	227 265 275 290

Both sub-fractions gave a partition ratio (95% MeOH : Hexane

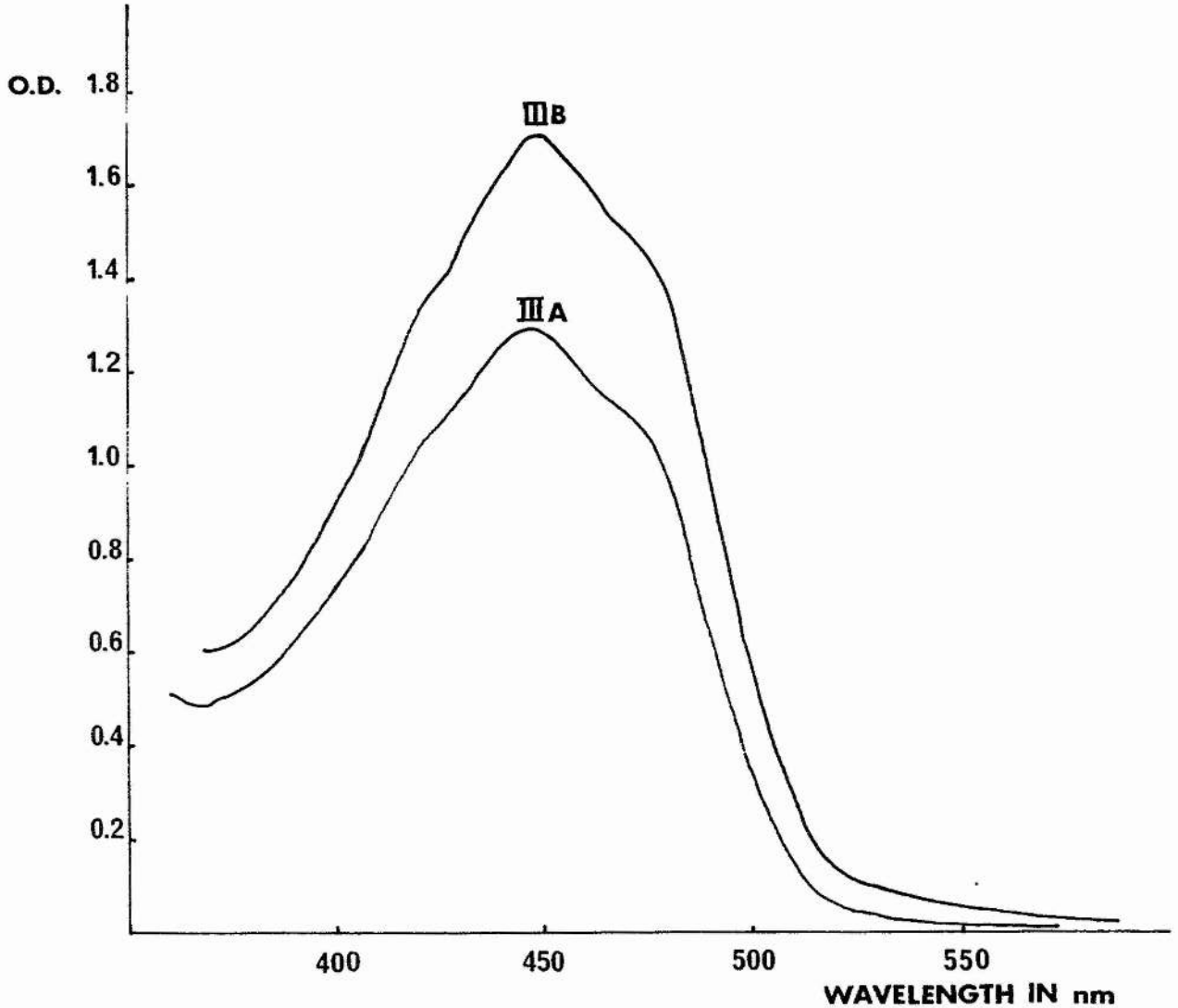


FIG. R.X Absorption spectrum of carotenoid sub-fractions IIIA & IIIB in methanol

of 100:0 and were not reduced by $LiAlH_4$.

Both sub-fractions were not degraded by saponification although there was only approximately a 50% recovery of the unsaponifiable material into the ether phase and as an orange-red precipitate at the interphase. The loss could be mainly accounted for by material in the aqueous phase which could not be recovered either into ether or into the precipitate at the interphase.

Thus from the etherial extract, we designate the two sub-fractions as III_{A1} and III_{B1} and from the precipitate at the interphase, we designate the two sub-fractions as III_{A2} and III_{B2}. All these sub-fractions appeared less polar than the original fraction when run on TLC but both their spectra in methanol and partition ratios were unaltered from the original III_A and III_B. Before hydrolysis, all four sub-fractions did not stain with anisidine phthalate but all did after hydrolysis in 1M HCl. This suggested the presence in each sub-fraction of carbohydrate which was initially linked in such a way that the reducing group of the sugar was involved. These acid hydrolysates were then prepared for G.L.C. as before and shown to contain three hexoses as shown in table R XXII.

Table R XXII G.L.C. analysis of hexoses in sub-fractions III_{A1}, III_{A2}, III_{B1} and III_{B2}

Fraction	% Galactose	% Glucose	% Mannose	Ratio
III_{A1}	76.6	15.6	7.7	10:2:1
III_{A2}	58.5	27.1	14.3	4:2:1
III_{B1}	67.2	16.2	16.7	4:1:1
III_{B2}	59.0	26.1	14.3	4:2:1

In this instance it was possible to calculate percentages since only the α galactose was detected (probably due to crystallisation of galactose during lyophilisation) and this avoided any overlapping of peaks as recorded by G.L.C.

Although aliquots of each sub-fraction containing equal amounts of carotenoid (as judged by their absorption at the λ_{max}), were hydrolysed in 1M HCl there was considerable variation in the quantity of monosaccharides liberated. The amount released was in the order $III_{A1} > III_{B1} >> III_{A2} > III_{B2}$. Each of these hydrolysates was also spot tested with ninhydrin and whereas III_{A2} and III_{B2} gave a strong ninhydrin colour, III_{A1} and III_{B1} gave only a weak ninhydrin reaction. All also showed λ_{max} consistent with the presence of amino acids/ peptides.

Thus, all four sub-fractions would seem to be mixed glycosides containing three monosaccharides in a given molar ratio, all linked through their reducing groups. It was not possible to determine the number of sugar residues per carotenoid molecule. The mixed glycosides are associated with peptides and it seems likely that larger peptides are associated with III_{A2} and III_{B2} and this may be responsible for inhibition of hexose release on hydrolysis. The structure of the complex is such that the chromophore of the carotenoid molecule is not significantly altered from that of the free pigments.

Characterisation of the water-soluble complex from S. aurantiaca

(1) Homogeneity

With both disc-gel electrophoresis (for 1-3½ hours) and attempted resolution on Sephadex G25 it was found that only one protein-containing fraction could be detected.

(2) Solvent Extraction

No pigment was partitioned from the aqueous phase into any of the various organic solvents tried. This suggested that the water soluble moiety of the complex was sufficiently large to maintain the entire complex preferentially soluble in the aqueous phase.

(3) Absorption spectrum

No fine structure was seen in the visible region of the spectrum. There was a marked increase in absorption towards the U.V. region from about 460nm to a plateau at 400 nm, and a further gradual increase in absorption to a broad peak at 225nm. The absence of fine structure in the visible region may suggest that when the complex is formed the normal chromophore of the free pigment is interfered with eventhough the complex had the same apparent colour as that of the free pigments in organic solvents (except carbon disulphide). It is more likely that the water-soluble complex contains a stubstantial peptide moiety and that the relatively high absorption of the latter in the U.V. region obliterates the relatively low absorption of the associated pigment within the visible range.

(4) Spot Staining

The presence of protein/peptide was shown by a strong reaction with ninhydrin. The complex did not stain with anisidine phthalate before hydrolysis with 1M HCl but did so after hydrolysis. The latter must therefore have released free reducing groups on the sugar moieties of the complex.

(5) GLC analysis of monosaccharides

The following hexoses were detected in the 1M HCl hydrolysate of the complex:- galactose (32.4%), glucose 21.8%) and mannose (45.7%). On the basis of the tests for homogeneity it is suggested that the complex is a mixed carotenoid glycoside associated with peptide/protein.

(6) Amino Acid Analysis

The results are shown in table R XXIII.

Table R XXIII Amino Acid analysis of the complex, expressed as moles/1000moles.

Amino Acid	M/1000M	Molar Ratio	
Cysteic Acid	11	2.8	(3)
Aspartic Acid	75	18.8	(19)
Threonine	44	11.0	(11)
Serine	83	20.8	(21)
Glutamic Acid	94	23.5	(23.5)
Proline	53	13.2	(13)
Glycine	141	35.2	(35)
Alanine	113	28.2	(28)
Valine	69	17.2	(17)

Table R XXIII continued

Amino Acid	M/1000M	Molar Ratio	
Methionine	Trace	-	-
Isoleucine	47	11.8	(12)
Leucine	105	26.2	(26)
Tyrosine	4	1.0	(1)
Phenylalanine	36	9.0	(9)
Lysine	38	9.5	(9.5)
Histidine	25	6.2	(6)
Arginine + Ornithine	62	15.5	(15.5)

Unknown small peaks were also recorded between phenylalanine and histidine and between valine and isoleucine.

Again, as with the whole membrane, there is a high proportion of apolar and acidic amino acids. Looking at the molar ratios of the individual amino acids, with the exception of glutamic acid and lysine, there are, within experimental limits, whole numbers again suggesting the homogeneity of the complex, and the presence of a single peptide.

(7) Determination of S^{o}_{20} value

On the analytical ultracentrifuge, homogeneity was again shown by the migration of a single symmetrical Schlieren peak. From the various photographs taken at periodic intervals of the migration of this peak, the S^{o}_{20} value of the material was found to be 2.35×10^{-13}. Assuming the complex to be mainly protein, and disregarding any effect on migration rate by either the carotenoid or carbohydrate moieties,

a comparison of this figure with those published for pure proteins would give an approximate molecular weight of 22,000. However, this value must be treated with caution since it may be totally unjustifiable to make a comparison between this material and a pure protein so far as S^{o}_{20} values are concerned. The use of interference optics on the ultracentrifuge would have permitted a more accurate determination of the molecular weight of the complex, but insufficient material was available for such work to be attempted.

DISCUSSION

The values of 28° as the optimum temperature for growth of S. aurantiaca agrees well with the figure of 30° quoted by Berghey (1957). The growth of S. aurantiaca is supported over a relatively wide temperature range from 20° (lowest reading) to 40° and similarly S. flava (Thirkell et al, 1965) and M. radiodurans (Thirkell, 1969) grow over wide ranges of temperature of 8 to 47° and 15° (lowest reading) to 40° respectively. Also, the growth of S. aurantiaca falls off steeply outside the maximum temperature range and this is similar to growth patterns of S. flava (Thirkell et al, 1965) and M. roseus (Thierry et al, 1966).

It was determined for S. aurantiaca that bacterial numbers are relatively constant from 50 hours to 70 hours of culturing (i.e. stationary phase) until readings were discontinued. This is concurrent with results reported for S. flava where no phase of decline was obvious up to 100 hours of culturing with the exception of the 40° culture (Thirkell et al, 1965). This contrasts with results reported for M. radiodurans where the bacterial number declined fairly rapidly after reaching their peak (Thirkell, 1969) and for S. maxima and S. ventriculi which are no longer viable after two to four days of incubation in liquid media, death probably being the result of the production of toxic metabolites (Canale-Parola, 1970).

The turbimetric and viable cell count techniques used for the determination of bacterial growth gave very similar results for the

growth of S. aurantiaca up to 70 hours of culturing. However, if readings are continued for a longer period, the latter method should be more accurate and indicate the phase of decline in the bacterial growth cycle.

Growth of S. aurantiaca was possible over the pH range of 5.5 to 8.5 with a pH optimum of 6.5 to 7.5. M. roseus also has a similar pH range for growth but this bacterium has two pH optima at 6.8 and 7.5 with decreased growth in between the two optima (Thierry et al, 1966). However, S. maxima and S. ventriculi both have a very wide pH tolerance of 1 to 9.8 (Canale-Parola, 1970) and this further demonstrates the differences between these bacteria and S. aurantiaca.

According to Goodwin (1963), for most bacteria the optimum temperature for pigmentation is lower than the optimum temperature for growth. This is true for S. flava (Thirkell et al, 1965) but not for S. aurantiaca, M. roseus (Thierry et al, 1966) and M. radiodurans (Thirkell, 1969) which have the same optimum temperature for both growth and pigmentation.

In most bacteria, the amount of pigment produced increases with age at least until the stationary phase of growth. This is the case for M. roseus (Thierry et al, 1966) S. aureus (Hammond et al, 1970) and for Myxococcus fulvus (Reichenbach et al, 1971). In S. flava, the maximum amount of pigment is produced early on in the growth phase, i.e. 18 hours after incubation and this maximum was maintained until

75 hours when it dropped to a steady value, (Thirkell et al, 1965). In S. aurantiaca, there is a different pattern of pigmentation with time. The peak of pigmentation is not reached until approximately 65 hours after the attainment of maximum cell numbers and is only maintained from approximately 90 to 95 hours of culturing at most temperatures. This value then declined up to 140 hours when the final determinations were made. The decline in pigmentation between 95 hours and 140 hours could be due to (a) turnover and degradation of the pigments (b) accumulation of storage compounds (granules) or more likely (c) to the presence of pigmentless cell debris as a result of autolysis during the decline phase.

The methods used for the extraction of carotencids and the types of carotenoids present in bacteria are important criteria when comparing the pigmentation patterns for S. aurantiaca with other bacteria. The relative amounts of the different pigments can alter throughout the growth phases and it has been reported that the polar carotenoid glycosides tend to accumulate in the stationary phase of growth (Aasen et al, 1969; Kleinig et al, 1971). Therefore, for a comparison of any results, it is essential to extract all the pigment, including any bound pigment, without incurring any degradation or loss of the carotenoids in the process. For bacteria such as M. roseus and S. aureas which do not contain any carotenoid glycosides, a total pigment extraction is easier and reproducible but the presence of these polar carotenoid

glycosides in many other bacteria can mean that the total pigment is not always extracted and thus gives anomalous results for their pigmentation pattern.

The percentage compsition of each membrane component in all three ages of S. aurantiaca membrane preparations is within the range of values reported for other Gram-positive bacterial membranes. However, whereas the overall quantitative chemical composition of the 27 hour membranes (early stationary phase) and the 57 hour membranes (late stationary phase) are similar, both are different from the 17 hour exponential phase membrane preparation.

All the membrane preparations were lyophilised prior to chemical analysis in order to obtain more accurate, reproducible results. After lyophilisation, the residual moisture content was quite high but remained constant throughout the investigation. As the moisture content can increase if the membranes are exposed to the atmosphere or decrease if storred for long periods in the deep freeze, it was important to check the moisture content throughout the investigation and this was done by monitoring their total phosphorus content at periodic intervals (Goodlad, 1970).

There was no loss of any coloured carotenoids, which, as specific membrane components, can be used as "markers", into the Tris buffer during the membrane isolation indicating that membrane components were not leaking out into the washing fluid. There did not appear to be any

cell wall contaminants present as indicated by the absence of rhamnose and the presence of only traces of hexosamines, both being typical cell wall components. Cytoplasmic contamination also appears to be negligible since the RNA content of the membranes is relatively low compared to that found in other bacterial membranes. Significant contamination of the membranes with the lytic enzyme was also avoided by the use of very low concentrations of enzyme in relation to the amount of membrane produced. Also, assuming that the enzyme is not bound to the membranes, most of it would be recovered in the supernatant when the membranes were centrifuged down as a pellet.

Total phosphorus determinations using the method of Allen were highly reproducible despite the low quantities of membranes used for the estimation. All the phosphorus results were typical for bacterial membranes although the phosphorus content of the 17 hour membranes (0.56%) was half the phosphorus content of the other membranes (1.16 and 1.015). A similar increase in phosphorus content with age is not shown by membranes of M. lysodeikticus (Salton et al, 1965) and the membranes of S. flava show a decreased phosphorus content from 1.2 to 0.7% with age (Hunter, 1971). The increase in the phosphorus content of S. aurantiaca membranes from exponential to stationary phases of growth may be due to either (a) increase in the RNA content with age (b) a 260% increase in the phospholipid content of the membranes from the 17 hour to the 57 hour preparations or possibly (c) to an increase

in polymetaphosphate granules contaminating the cell membrane preparations since polyphosphate is known to be a common cytoplasmic inclusion in Gram-positive bacteria (Friedberg and Avigad, 1968).

The RNA contents of the 27 hour and 57 hour membranes (1.49 and 1.99%) are comparable with the percentage RNA found in some other bacterial membranes (see table I. 1. in the Introduction) but the 17 hour membranes have a low RNA content of 0.33% and since few workers have published results for exponential phase membranes, it is not known whether this low value is peculiar to S. aurantiaca. Salton et al, (1965)also found an increase in the RNA in the membranes of M. lysodeikticus from 24 hours to 48 hours but Hunter (1971) reported that the RNA in S. flava membranes was highest in the exponential phase and fluctuated in the stationary phase.

The Moore and Stein method for protein estimation gave the most consistent results for bacterial membranes. It can be assumed that there was only minimal interference from hexosamines because there were only trace amounts of these compounds present in all of the membrane hydrolysates. Other methods of protein estimation e.g. Biuret (which is not sensitive enough), Folin-Lowry (which depends on the presence of significant amounts of aromatic amino acids, which are known to be present in low concentrations in bacterial membranes) and total nitrogen (which also measures nitrogen from non-protein sources) all have some disadvantages when applied to bacterial membranes. This is partly why such wide variations in the amount of protein in membranes have been reported when different assay

techniques have been used.

The protein content of S. aurantiaca membranes remained approximately constant with increasing age of the culture and this is consistent with results reported by Salton et al, (1965) for M. lysodeikticus. However, the membranes from S. flava (Hunter, 1971) and S. faecalis (Shockman et al, 1963) appear to have a decreased membrane protein content from the exponential to the stationary phase of growth. If the majority of the protein is of the structural type, one would expect comparatively little variation with age but if a large proportion of the protein is enzymic, more variation could be expected since the enzymes may well change with age of the culture to accomodate the different processes occurring in the cell throughout the growth cycle.

The methods used for extracting protein components from S. aurantiaca membranes were not very successful and this was probably because most of the protein may well exist in the membrane as lipo-protein complexes which are not easy to disrupt. As the protein component of the membrane should be heterogeneous including several enzyme fractions, the protein fractions isolated by different methods could be lipoproteins and this could account for the identical mobilities and lack of resolution on disc gel electrphoresis of the fractions isolated by the different methods. This would seem to be

confirmed by the fact that most extraction methods found in the literature employ organic solvents and virtually appear to treat the protein as hydrocarbons.

The amino acid composition of all three membrane hydrolysates were similar to the amino acid patterns of many other bacterial membranes and to cell membranes in general, in that there were large amounts of apolar and acidic amino acids together with low amounts of basic amino acids. The presence of a large ammonia peak is suggestive of a large proportion of the acidic amino acids being present in the amide form as postulated by Grula et al (1967) for M. lysodeikticus membrane protein. The large amount of apolar amino acids suggests a high degree of hydrophobic bonding but the possible presence of acidic amino acids in the amide form could indicate ionic linkages to the phosphate groups of the phospholipids.

Although the overall percentage of protein is S. aurantiaca membranes was constant, there were variations in the amino acid ratios from the exponential to the stationary phases of growth. The most marked changes were a large increase in alanine with smaller increases in lysine and methionine from the exponential to the stationary phase of growth, concomitant with a compensatory decrease in the amount of all the other amino acids. Since, the amino acids are not present in exact molar ratios in any of the membrane preparations

several proteins must exist in the membrane. Again, the variations in the amino acid values may be the result of a changing pattern of such protein components with age. Also, the murein peptide of S. aurantiaca cell wall is of the L-lysyl-L-alanyl$_3$ type (Morrison et al, 1971) and it is possible that the cell wall precursors accumulate in the membranes in the stationary phase of growth if the cut off mechanism for cell wall synthesis is not very efficient.

The phenol-H_2SO_4 method was used for estimation of carbohydrate because it measures total carbohydrate (hexose + pentose) of the membranes and is not subject to interference from other membrane components. This method is not very accurate if large quantities of hexose and pentose are present since the hexose absorption maxima is at 490 nm and the pentose at 480 nm and a large extinction overlap would grossly increase the results. However, the ribose content of S. aurantiaca membranes was low compared to the hexoses and thus the readings obtained should approximate to the total carbohydrate present. According to Whistler et al, (1962) the anthrone method is subject to interference from protein and was therefore not used for this investigation because of the large amount of protein present in the membranes. It is thus surprising, that a number of workers have used the anthrone method for estimation of the carbohydrate content in bacterial membranes (see Table I. II in the introduction).

The total carbohydrate in S. aurantiaca membranes increased from 7.19% in exponential phase to 11.10% in late stationary phase, an

increase not found in S. flava membranes (Hunter, 1971). This increase may be partly due to an increased RNA content and it is not clear what contribution the glycolipid has towards the total carbohydrate since the proportion of this lipid fluctuates slightly during the growth cycle. The increase in carbohydrate content could also be due to other carbohydrate components in the membranes e.g. oligosaccharides, polysaccharides or glycopeptides since analysis of the glycolipid carbohydrate indicated that not all the membrane carbohydrate was accounted for as glycolipid or RNA. This was further substantiated by the observation that the glycolipid in the 17 hour culture contains only trace amounts of the glucose whereas the total membrane hydrolysate has a relatively high glucose content.

Mannose, glucose, galactose and ribose were detected in all three membrane hydrolysates and these sugars were also found in membranes of S. lutea and M. lysodeikticus (Salton et al, 1965b). However, there is no species specificity for these sugars since S. flava contained rhamnose and no galactose (Hunter, 1971). The ratio of the individual hexoses in S. aurantiaca membranes varies with age, some glucose being replaced by galactose from the exponential to the stationary phases of growth. As expected, only trace amounts of glucosamine and galactosamine were detected by GLC.

100% of the components of the 17 hour membranes were accounted for by the analyses but for the 27 hour and 57 hour membranes only 88.6%

and 86.6% of the constituents could be accounted for. This discrepancy in recovery could be due to an incomplete extraction of the lipid since it is surprising that the lipid component should markedly decrease from 39.7% to 19.30% with age whilst the other main component, protein, remains constant. S. flava membranes also exhibit a decreasing lipid content with age but many other bacterial membranes show an increase in lipid with age (O'Leary, 1967). The decrease in lipid content for S. aurantiaca membranes may merely reflect the limitations of the extraction methods used as it is probable that more lipid becomes complexed with other membrane constituents, particularly protein, as the culture ages and this bound lipid would be more difficult to extract without incurring any degradation.

The other main membrane component in the "in vivo" state is water and it was found to be necessary to soak the membranes in water prior to solvent extraction in order to extract the maximum amount of lipid. Other workers who have extracted lipids from bacterial membranes of closely related species have used heat in order to extract the maximum quantity of lipid (Cho et al, 1966; Tornabene et al, 1967; Hunter, 1971). Therefore, it was surprising that the second method used in this work extracted more lipid from S. aurantiaca membranes despite the fact that heat was not used. It could be that increased agitation of the suspension on the magnetic stirrer brought the membrane particles into closer contact with the

extracting solvent or it could merely be a reflection of the different ratios of lipid classes found in these membranes. There is a further possibility that the reflux conditions may degrade some lipids to release volatile material which is then lost.

The membranes were also hydrolysed with methanolic HCl and finally with 2N KOH in order to extract the complexed lipid. However, these procedures, particularly the latter, will also cause the formation of some water soluble degradation products but do not effect the fatty acids (O'Leary, 1967). The decrease in free lipid and the corresponding increase in the acid hydrolysed lipid with age is indicative of an increased binding of the lipid in the membrane with age as found by Hunter, (1971) for S. flava. This complexing of the lipid is also shown by the observed decrease in the neutral lipid and the corresponding increase in the phospholipid with age.

The amount of neutral lipid in S. aurantiaca membranes is very high compared to many other bacterial membranes which often contain a large proportion of phospholipids (see Table I. III in the introduction), but S. lutea (Huston et al, 1964) and S. morrhuae (Hunter, private communication 1971) have also been shown to contain a large percentage of neutral lipid. It is already known that 20% of the lipid is hydrocarbon in nature (Morrison et al, 1971) and the remainder of the neutral lipid (46-50% of the total lipid) may be glycerides found in a high proportion in S. lutea (Huston et al, 1964) and free fatty acids.

The high neutral lipid content should not be due to any degradation of the phospholipid in the extraction procedure since very mild conditions were used but there is a small possibility of degradation of some phospholipid to neutral lipid during storage (Ghosh et al, 1967). Also, although the possible influence of endogenous phospholipases must be considered, under the conditions used during this work, their effect must be minimal. The observed results for the phospholipid content could be low since there might be phospholipid complexes in the membrane which are not extracted or degraded by the methods used.

The glycolipid fractions contained mannose, galactose and glucose with mannose predominating. A dimannosyl diglyceride has been isolated from M. lysodeikticus (Lennarz and Talamo, 1966) and since closely related species have identical glycolipids (Shaw et al, 1968) the glycolipid fraction III could well be this dimannosyl diglyceride since it contained almost exclusively mannose as the carbohydrate moiety. Glycolipid fraction II could be a mixture of monoglycosyl diglycerides or a mixed diglycosyl diglyceride containing two different hexoses and could also include some acylated sugar derivative since they are also frequently occurring bacterial glycolipids (Shaw, 1970).

The fatty acids found in S. aurantiaca membrane lipids are typical for Gram-positive bacteria in that they contain a high proportion of branched chain fatty acids, iso and anteiso, even and odd numbered, together with only a very small percentage of unsaturated fatty acids.

However, they also contain significant amounts of straight chain, even numbered fatty acids e.g. palmitic acid and stearic acid which are normally found in large quantities in Gram-negative or Gram-variable and anaerobic bacteria. However, S. lutea cells (Huston et al, 1964) and S. flava membranes (Hunter, 1971) also appear to contain significant amounts of straigh chain, even numbered fatty acids together with a large proportion of branched chain fatty acids.

Morrison et al, (1971) reported that the fatty acids of S. aurantiaca are similar to other Micrococci. They analysed the fatty acids of S. aurantiaca cells grown to early stationary phase in trypticase soy broth. Since, the medium was different from the one used in this investigation, the results are not necessarily comparable as it is known that the relative amounts of fatty acids synthetised varies according to the composition of the medium (Tornabene et al, 1967). In addition there might be slight differences due to the fact that Morrison et al, (1971) analysed the cell lipid and this investigation was concerned with the membrane lipid of S. aurantiaca. However, these workers do state that the major fatty acid present is the branched chain C_{15}, and that saturated, branched chain and straight chain, even and odd numbered fatty acids were also found, although no indication was given as to the relative amounts of the individual fatty acids present.

Tornabene et al, (1967) found no significant variation in the fatty acids during growth of S. lutea cell but Hunter (1971) working

with S. flava membranes, reported fluctuations in the quantity of several fatty acids, particularly the branched chain C_{15} with age of the culture. He found that the free lipid and acid hydrolysed lipid fraction (majority of the lipid) of the 57 hour (early stationary phase) membranes had a much decreased content of the branched chain C_{15} fatty acid compared with the 24 hour (exponential phase) and the 91 hour (late stationary phase) membranes.

This decrease in the branched chain C_{15} fatty acid in the early stationary phase culture is even more marked for S. aurantiaca membrane lipids. The free lipid and the acid hydrolysed fractions (most of the lipid extracted by method I) and the neutral lipid fraction (large proportion of the lipid extracted by method II)all showed this decrease in the level of the branched chain C_{15} fatty acid, concomitant with an increase in palmitic acid and iso and anteiso branched chain C_{19} fatty acids. There is no obvious reason for this change in the fatty acid pattern but it could be the cause of a permeability change in the membranes at the onset of the stationary phase of growth as the cells adapt to the exhaustion of a particular nutrient and/or the build up of metabolites.

There were no obvious differences in the fatty acid patterns between the glycolipids and the phospholipids for all three membrane preparations and this is in agreement with results obtained by Shaw (1970) for other bacterial lipids. The branched chain C_{15} fatty acid

predominating in all these complex lipid fractions and this fatty acid is also the major fatty acid of the complex lipids of S. lutea (Huston et al, 1964). It can be seen that there are more variations in fatty acids with age in the neutral lipid fraction than the other fractions. The more marked fluctuation in the fatty acid composition in the neutral lipid fraction as compared with the complex lipid fractions could be partly due to variation in the more easily exchangeable free fatty acids in this fraction in contrast to the more stable esterified fatty acid moieties of the glyceride derivatives.

This investigation has demonstrated a high turnover of fatty acids in the membrane which causes complications when attempting classification on the basis of fatty acid composition but generally, it can be said that the fatty acids present in S. aurantiaca were typical of a Gram-positive bacterium.

The membranes of S. aurantiaca have a comparatively higher lipid : protein ratio and a higher carbohydrate content than many other bacterial membranes. The low percentage of phospholipid present and the large amount of neutral lipid suggests that the latter may well be involved in hydrophobic bonding with the apolar amino acids of the membrane protein. The phospholipid is also probably involved in ionic linkages with the amides of the acidic amino acids (if present, as suggested by the large ammonia peak). The glycolipid is postulated by

Shaw (1970) to be involved in pore formation, whereby the apolar areas are directed towards the interior and the polar hydroxyl groups of the glycolipid come together to form a hydrophilic pore. If this is correct, it could be an explanation for the decreased percentage of glycolipid in the late stationary phase culture when the cell nutrient requirement must be reduced.

The lack of cysteine residues means that the absence of disulphide bridges would confer more flexibility on the protein layer. The lipid in S. aurantiaca membranes also has more flexibility because the high proportion of branched chain fatty acids means that the fatty acids are not closely packed together (Kodicek, 1962). The general flexibility of the protein and lipid components would give the membrane considerable permeability properties.

In conclusion, it may be said that Gram-positive bacterial membranes are a useful source for the study of cell membranes. Bacteria can be grown in quantity and their membranes are relatively easy to prepare in a "pure" state and consist only of the cytoplasmic membrane and mesosomal membranes. In contrast, the membranes of an eucaryotic cell may well be more diverse since in addition to the actual outer membrane of the cell, there are several other membranes which are derived from the different membrane bound sub-cellular organelles.

It is well known that the carotenoids are very unstable in the presence of light, oxygen, heat and acid. Most of them are also stable in the presence of methanolic alkali but some exceptions to

this are known e.g. fucoxanthin, some phenolic carotenoids and oscillaxanthin. During the course of this work, it was realised that at least some of the carotenoid fractions of S. aurantiaca were also unstable to saponification. The same pigments also appeared to be degraded by certain extraction techniques e.g. ultrasonication or the use of the Vibrogen cell mill where local heating may be involved. In the latter cases, artefacts were produced with markedly altered chromophores, including some which were blue. When these artefacts were examined for the presence of epoxy or furanoid oxides, as expected, it was found that these were not present and that the gross spectral change must thus be due to degradation. Such degradation is not shown under these conditions by the carotenoids which have been extracted from other bacteria in this laboratory.

Therefore it was important from the outset, to use a suitable carotenoid extraction procedure which would effect a total pigment extraction, particularly of the polar complexed pigments found in S. aurantiaca, without the production of any degradative artefacts.

The extraction of pigments from S. aurantiaca membranes using chloroform : methanol (2:1) v/v had several advantages, the most important being that it gave a quantitative pigment extraction of the cell membranes from a 27 hour culture leaving the remnants colourless, without incurring any degradation products. The absence of artefacts using this method is almost certainly due to the lack of local heat production as is inevitable in the case of ultrasonication and ballistic disintegration.

The extraction was carried out at 4°, in the dark, and under an atmosphere of nitrogen to minimise any possible degradation. The absence of cis isomers in the carotenoid fractions further proved the advantage of using this method since most naturally occurring carotenoids occur in the all-trans form. Membranes were prepared prior to pigment extraction since the outer cell wall was shown to prevent an efficient extraction with organic solvents.

To the author's knowledge, only the carotenoids of S. aureus have previously been isolated using a chloroform : methanol mixture in the absence of heat when Hammond et al (1970) utilised a modification of the Bligh and Byer procedure. Total pigment extraction from S. aureus by such a procedure is not surprising since S. aureus has not been shown to contain any carotenoid glycosides or glycopeptides. However, in S. aurantiaca, at least 71% of the total pigment consists of carotenoid glycopeptides. Therefore it was unexpected that the above method should extract all the pigment from S. aurantiaca.

As one would expect, the initial pigment extract included a high percentage of other lipids which were difficult to eliminate completely. The lipid precipitation technique of Blessin removed a portion of the other lipids but the best purification procedure, saponification in 0.5% (w/v) methanolic KOH could not always be used due to the instability of some of the pigment fractions to this process. Saponification is a widely used purification technique for

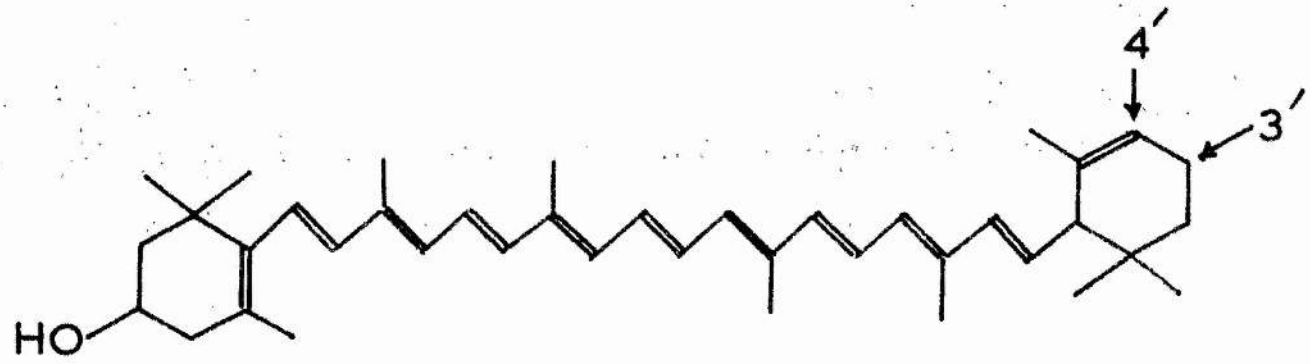
carotenoids except in the case of the few carotenoids already mentioned as being unstable to alkali.

The high polarity of the majority of the pigments from S. aurantiaca caused difficulties with the selection of both a suitable adsorbent and a suitable solvent system for use in column chromatography or TLC. Column chromatography using activated silicic acid which separates carotenoids according to the type and number of oxygen fractions, proved to be the most efficient initial method of separation. Subsequent purification of the column fractions was then easily carried out by TLC on silica gel G (Merck) as adsorbent. The solvent system Benzene : methanol : acetic acid (87:11:2) v:v:v produced an adequate separation by TLC on silica gel G (Merck) plates but again artefacts were produced almost certainly due to the presence of acid. Nevertheless, this separation technique was used by Strang (1968) to purify several bacterial carotenoids.

All the carotenoid pigments isolated from S. aurantiaca were shown from their spectra to be related to α-carotene. This is unusual since α-carotene derivatives are uncommon in bacteria although they occur frequently in plants and algae (Liaaen-Jensen, 1970). However, 4% of the total carotenoids of M. roseus were shown to be α-carotene derivatives (Ungers and Cooney, 1968). Although published data on the majority of α-carotene derivatives show spectra with detailed fine structure, a dihydroxy-α-carotene from M. roseus did

not have a well defined visible absorption spectrum. The visible absorption spectra of the more polar S. aurantiaca carotenoids were also not well defined even though carbonyl, aldehydic and acid groups which can cause loss of fine structure were proved to be absent. Ester groups, tertiary and allylic hydroxyl groups and methyl substitutions were also undetected.

For most bacteria, the carotenoids are synthesised by a biosynthetic sequence in which the oxygen functions are inserted at a terminal stage after the production of the fully unsaturated hydrocarbon (Goodwin, 1963). This work has shown that, for S. aurantiaca, the parent hydrocarbon of the series of carotenoid pigments is almost certainly α-carotene (Fraction I_A) and that the monohydroxylated derivative zeinoxanthin (Fraction I_B) is then produced. The evidence for the identity of the dihydroxylated compound (Fraction II) is rather more tentative, but preliminary indications that it might be lutein were not substantiated since it is unstable to alkali, allylic hydroxyl groups are absent and a purple degradation product is formed with NiO_2. Since the first hydroxyl group is substitued on the β-ionone ring (zeinoxanthin), it is most likely that the second hydroxyl group is substituted on the α-ionone ring. The hydroxyl group is not substituted on the 3' position (lutein) which leaves the 2' and 4' positions. The 4' location next to the double bond seems more probable as this could possibly account for the instability of this fraction as compared with Fraction I_B.



Comparatively little work has been carried out by other workers on the pigments of S. aurantiaca. Reports by Reader (1952), Chargaff (1933), Sobin et al, (1942) and Strang et al (1969) are very inconclusive and contradictory. Sobin et al, (1942) and Strang et al, (1969) both used an extraction and purification procedure which involved heat and saponification, both of which have been shown by the author to result in the degradation of some of the carotenoids. It is therefore not surprising that they reported different results from those presented in this work.

Sobin et al, (1942) reported the presence of nine coloured carotenoids in S. aurantiaca including two carotenols and the absorption maxima of one or more of the pigments were close to those to zeaxanthin. Also, Strang et al (1969) claimed to have found β-carotene and zeaxanthin in S. aurantiaca when comparing these with pigments of other bacteria, but they do not present any evidence for this assumption.

The differences between the visible absorption maxima of α and β carotenes are not very large unless spectra are recorded in solvents such as chloroform or carbon disulphide. Errors can also arise, if the solvents used are impure. Some of the nine pigments found by Sobin et al, (1942) could be artefacts formed during isolation and also during saponification. According to Liaaen-Jensen (1970) lutein will isomerise to zeaxanthin under strong alkaline conditions and it is therefore possible that the dihydroxy carotene fraction from S. aurantiaca will also isomerise under the conditions employed during saponification. Early investigations by the author using 10% (w/v) methanolic KOH did produce a zeaxanthin-like fraction and it is postulated that this is why previous workers have reported zeaxanthin as a carotenoid found in S. aurantiaca.

The carotenoid glycopeptides in S. aurantiaca form 71.4% of the total pigment fraction of a 27 hour culture (stationary phase of growth) and this would be expected if these carotenoid glycopeptides are the terminal product of the biosynthetic pathway. Carotenoid glycosides in some other bacteria also form the major part of the total pigment, (Table I. IV). In analogy to other bacterial carotenoid glycosides, the aglycone is probably the most polar free carotenoid, the dihydroxy α carotene derivative and since the latter is unstable to saponification, this may explain why 50% of the glycopeptide fraction is lost during saponification.

Fraction III (the carotenoid glycopeptide derivative) could only be resolved into sub-fraction III_A and III_B by TLC using the solvent system 0.5% (w/v) pyridine in methanol on silica gel G (Merck) plates. Many difficulties were encountered when investigating these fractions since they were initially associated with significant amounts of other lipids. To remove these lipids, saponification had to be used even though a 50% loss in carotenoid material occurred. After saponification the ether-soluble sub-fractions III_{A1} and III_{B1} and the ether-insoluble fraction III_{A2} and III_{B2} did not exhibit altered chromophores i.e. they gave the characteristic visible absorption spectra of an β-carotene derivative containing a chromophore of ten conjugated double bonds.

The partition ratios of all four sub-fractions before and after saponification indicated the presence of three or more hydroxyl groups and the absence of carbonyl groups was confirmed for all the fractions. It was impossible to acetylate these fractions (reasons presented in the "Methods section") and this prevented the determination of the exact number of hydroxyl groups present and also any possible further resolution of these carotenoid glycopeptides in the acetate form. In this connection, it is worth noting that Hertzberg et al, (1967) only managed to separate the glycosidic phleixanthophyll and 4-keto-phleixanthophyll after acetylation.

The hexoses galactose, glucose and mannose were detected using GLC in all of the carotenoid glycpeptide fractions although the relative

amounts of the individual monosaccharides differed. Both ether-soluble fractions contained the hexoses in the ratio galactose : glucose : mannose :- 4:2:1 whilst for III_{A1} the ratio was 10:2:1 and for III_{B1} 4:1:1. Using the anisidine phthalate spray reagent, no sugars were detected until after hydrolysis in 1M HCl showing that the reducing groups of all the sugar molecules were involved in glycosidic linkages, probably with carotenoid, or with other sugar moieties, or with amino acids. The possibility of the hexoses being linked to either serine or threonine through O-serine or O-threonine linkages can be discounted. The material had been saponified in 5% methanolic KOH which is a higher alkali concentration than is usually required to split such links, by β-elimination.

All four sub-fractions gave a positive ninhydrin reaction proving that amino acids were present and possibly attached to the carotenoid glycoside, esterified to a free hydroxyl group on a hexose moiety as suggested by Thirkell et al (1969). It is postulated that the ether-soluble fractions must contain only a few amino acid residues (weak ninhydrin reaction) and that the other sub-fractions must contain longer peptides thus making these fractions ether-insoluble but still soluble in methanol.

Sub-fractions III_{A2} and III_{B2} were soluble in light petroleum (60-80°) before saponification but were insoluble after saponification

and this is probably due either (1) degradation of lipid previously occluding the carotenoid glycopeptides or (2) to release of fatty acids previously esterified to hydroxyl groups on the hexose moiety since carotenoid glycosides containing esterified fatty acids, have been isolated from several myxobacteria, the first being discovered in Stigmatella aurantiaca by Kleinig et al, (1970). Similarly the different mobilities of sub-fractions III_A and III_B on silica gel G (Merck) could be due to (a) associated lipid impurities or (b) to esterified fatty acids or (c) to differences in the relative amounts of the individual hexoses and/or amino acid components of the complexes.

The water-soluble carotenoid containing fraction isolated from S. aurantiaca using a different method to the technique used for the isolation of sub-fractions III_A and III_B, contained the same hexoses again linked through their reducing groups. However, this time the hexoses were in the ratio of galactose : glucose : mannose : 1.5:1:2 homogeneity tests using sephadex G_{25}, disc gel elctrophoresis and the analytical ultracentrifuge all indicated that this complex was homogeneous despite the inclusion of the three different hexoses. Such homogeneity was further indicated by the fact that amino acid analysis of hydrolysates of the complex showed that almost all (13 out of 15) of the individual amino acids were present in exact molar ratios. It was also shown that the amino acid composition of the peptide/protein moiety of the complex was similar to the composition of the whole membrane proteins.

This complex is water soluble and could not be partitioned into organic solvents. It is postulated that the difference between this fraction and the other carotenoid glycopeptide fractions is the presence of larger peptides/proteins making the fraction preferentially water soluble. This is further substantiated by the fact that the visible absorption spectrum of this very polar fraction is obscured by the very high UV absorption. Nevertheless, there is no evidence of any interference with the chromophore since the colour of the fraction to the naked eye is similar to that of the free pigments.

The figure of 20,000 obtained for the molecular weight of the complex is only very approximate as the complex is not pure protein. The only other water-soluble carotenoid glycopeptides so far reported were isolated by Thirkell et al (1969; 1970) from S. flava and S. morrhuae.

The carotenoid glycopeptides from S. aurantiaca are the only compounds containing carotenoid and hexose in which three different hexoses are found. Nearly all the carotenoid glycosides isolated contain only one type of sugar, the only exception being myxoxanthophyll. The evidence presented by Hertzberg et al (1969) suggests that this chromatographically homogeneous glycoside is, in fact, a mixed glycoside containing rhamnose as the dominant sugar moiety (90%) and a hexose as a minor component (10%). They could not separate myxoxanthophyll into any further components even after acetylation. Kleinig, Reichenback, Achenbach and Stadler, (1971) found both carotenoid

glucosides and carotenoid rhamnosides in Scrangium compositum but they were able to separate these different glycosides by column chromatography.

Liaaen-Jensen, Weeks Strang and Thirkell (1967) and Thirkell et al, (1967) have reported that C_{50} carotenoids are found in Gram-positive, non-photosynthetic bacteria, but it is only possible to distinguish between C_{40} and C_{50} carotenoids using mass spectrometry. It would have greatly facilitated the present investigation, if mass spectromety and PMR techniques had been available, particularly for the investigation into the carotenoid glycopeptide fractions from S. aurantiaca.

This investigation into the pigments of S. aurantiaca verified the earlier observations by Strang et al, (1969) that these carotenoids differ from those found in the related species S. flava, S. lutea and M. lysodeikticus. Altogether, it is obvious that carotenoids have no value in taxonomy since the Micrococci contain different types of carotenoids.

SUMMARY

The optimum temperature for both growth and pigmentation of S. aurantiaca was found to be 28°. Growth of S. aurantiaca was limited above 40°. Pigmentation was maximal 65 hours after maximum bacterial numbers were obtained, but began to decline rapidly. Growth only occurred to any substantiated degree within narrow pH limits.

The total membrane fraction from S. aurantiaca cells harvested at 17 hours (exponential phase), 27 hours (early stationary phase) and 57 hours (late stationary phase) were analysed to determine if any change occurred in the chemical components of the membranes from the exponential to the late stationary phases of growth. The percentage composition of the membrane components in all three membrane fractions were within the range of values reported for membranes isolated from other Gram-positive bacteria. Membranes from the two stationary phase cultures had a similar quantitative chemical composition but both differed in several respects from the membranes isolated from the exponential phase culture.

The protein content remained approximately constant throughout the growth phases but the lipid content decreased and the carbohydrate content increased, with age. Since the overall recoveries of organic material from the total membranes decreased with age, it is thought that this could be explained by increased binding of the lipid to protein which would mean decreased lipid extraction and anomalously

low lipid contents. This is substantiated by the observed increase in the relative amounts of the bound lipid and phospholipid fractions with age. Analysis of the lipid showed the presence of an unusually high amount of neutral lipid and a low quantity of phospholipid in all three membrane preparations, as compared with those found in other membranes.

The fatty acids found in all the membrane hydrolysates were generally typical for a Gram-positive bacterium but there were considerable variations in the relative quantities of the individual fatty acids with age. Amino acid analysis indicated an amino acid content similar to membranes isolated from other sources, and there were again variations in the molar ratios of the amino acids with age.

The monosaccharides detected in all the membrane preparations were galactose, glucose, mannose and ribose. The hexoses were shown to be constituents of the glycolipid and carotenoid glycopeptide fractions. Only trace amounts of galacatosamine and glucosamine were found.

All the carotenoids detected in a 27 hour culture of S. aurantiaca were shown to be derived from α-carotene. The free pigment consisting of α-carotene, zeinoxanthin and a dihydroxy-α-carotene formed approximately 29% of the total pigment. The remaining 71% were

carotenoid glycopeptides where the carotenoid is most probably linked to galactose, glucose, mannose and to amino acids/peptides. The hexose moieties were linked to carotenoid and/or peptides or other hexose moieties by glycosidic bonds involving their reducing groups.

Amino acid analysis of a water soluble carotenoid glycopeptide indicated an amino acid composition similar to the total membrane protein. This carotenoid glycopeptide appeared to be homogeneous despite the inclusion of three different hexoses. S^{o}_{20} values of the carotenoid glycopeptide indicated a molecular weight of the order of 20,000.

APPENDIX

During the early stages of this work, a major contamination problem was encountered when bacteria were grown in the New Brunswick MF 114 fermentor. Even though it was shown that the medium had been successfully sterilised in the autoclave by aerating it without inoculation for several days without any growth occurring, and that the inocula were pure by plating out on agar plates and microsopic examination, on inoculation of the medium, cultures of S. aurantiaca apparently grew normally for about 12 hours after which the entire culture was overgrown by a spore-bearing Bacillus. Subsequent investigation by the author and help from the Bacteriology department in Dundee failed to eliminate this problem. After a few month the contamination disappeared suddenly. Later discussions by Dr D. Thirkell with workers elsewhere suggested that heat resistant spores may have lodged in the oil seal and bearing of the impeller, and that once cultures grew to the point where foaming was produced at the surface of the culture, this foam could reach into the impeller bearing and bring down into the culture the vegetative form of the organism which then overgrew the culture. It is suggested that should such contamination be encountered again, the best corrective procedure should be to strip the impeller bearing and clean this out and also to renew at least the bottom oil seal.

BIBLOGRAPHY

Aasen A. J., Francis G. W., Liaaen-Jensen S. Acta. Chem. Scand. 23, 2605, 1969

Abel K., Schmertzing H., Peterson J. I. J. Bact. 85, 1039, 1963

Akáshi S., Saito K. J. Biochem (Tokyo) 47, 222, 1960

Allen R.J.L. Biochem J. 34, 858, 1940

Baird-Parker A. C. J. Gen. Microbiol 30, 409, 1963

Baird-Parker A. C. J. Gen. Microbiol 38, 363, 1965

"Bergey's Manual of Determinative Bacteriology", edited by Breed R.S., Murray E.G.D., Smith N. R., 7th edition, 1957, E. and S. Livingstone Ltd., (Edinburgh and London)

Blessin C. W. Cereal Chem. 39, 236, 1962

Bohacek J., Kocur M., Marinec T. J. Gen Microbiol 46, 369, 1967

Booth, V. H. Analyst 84, 464, 1959

Brown J. W. Biochim. Biophys. Acta 52, 368, 1961

Burchfield H. P., Storrs E. E. In "Biochemical Applications of Gas Chromatography" Academic Press (New York and London) p. 549, 1962

Canale-Parola E. Bact. Revs. 34, 82, 1970

Chargaff E. Compt. Rend. 197, 946, 1933

Cheesman. D. F., Lee D. F., Zagalsky P. F. Biol Revs. 42, 131, 1967

Cho K. Y., Salton M.R.J. Biochim. Biophys. Acta 116, 73, 1960

Cowan S. T. J. Appl. Bact. 25, 324, 1963

Crounse J., Feldman R. C., Clayton B. K. Nature 198, 1227, 1963

Daron H. L. J. Bact. 101, 145, 1970

Eberhardt U. Arch. Microbiol 80, 32, 1971

Estrugo S. F., Larraga V., Corrales M. A., Duch C., Munoz E. Biochim. Biophys. Acta 255, 960, 1972

Folch J., Lees M., Sloane-Stanley G. E. J. Biol. Chem. 226, 497, 1957

Friedberg I., Avigad G. J. Bact. 96, 544, 1968

Ghosh B. K., Carroll K. K. J. Bact. 95, 688, 1968

Ghosh B. K., Murray R.G.E. J. Bact. 97, 426, 1969

Gilby A. R., Few A. V., McQuillen, K Biochem. Biophys. Acta. 29, 21, 1958

Gillam A. E., Stern E. S. "An Introduction to Electronic Absorption Spectroscopy in Organic Chemistry". Edward Arnold, London 1955

Girard A. E. Can. J. Microbiol 17, 1503, 1971

Goodlad G.A.J. Private Communication, 1970

Goodwin T. W. Biochem. J. 63, 481, 1956

Goodwin T. W. "Biosynthesis of Vitamins and Related Compounds" Academic Press London and New York 1963

Goodwin T. W. "Chemistry and Biochemistry of Plant Pigments" Edited by Goodwin T. W. Academic Press, London and New York. 1965

Goodwin T. W. "Carotenoids" edited by Isler O., Gutmann H., Solms U., Berkhauser Verlag Basel, p. 577, 1971

Griffiths M., Sistrom W. R., Cohen-Bazire G., Stanier R.Y. Nature 176, 1211 1965

Grula E. A., Butler T. F., King R. D., Smith G. L. Can. J. Microbiol 13, 1499, 1967

Haidak D. J., Mathews C.K., Sweeney B. M. Science 152, 212, 1966

Hammond R. K., White D. C. J. Bact. 103, 191, 1970

Heptinstall S., Archibald A. R., Baddiley J. Nature 225, 519, 1970

Hertzberg S., Liaaen-Jensen S. Acta Chem. Scand. 21, 15, 1967

Hertzberg S., Liaaen-Jensen S. Phytochemistry 8, 1281, 1969a

Hertzberg S., Liaaen-Jensen S. Phytochemistry 8, 1259, 1969b

Hunter M.I.S. Ph.D. Thesis, University of St Andrews 1971

Hunter M.I.S. Private Communication 1971

Hunter M.I.S., Thirkell D. J. Gen. Microbiol 65, 115, 1971

Huston C. K., Albro P. W. J. Bact 88, 425, 1964

Jeffrey E. G. Private Communication, 1970

Jensen S. L. Ph.D. Thesis, I Kommisjon Hos. Trondheim 1962a

Jensen S. L. Kgl. Norske Videnskab. Selskabs, Skrifter 1962b No. 8

Jensen S. L. In "Carotenoids" edited by Isler O., Gutmann H., Solms U. Berkhauser Verlag Basel, P 61, 1971

Jensen S. L., Jensen A. In "Progress in the Chemistry of Fats and Other Lipids" Vol. VIII Pergamon Press, P 129 1965

Jensen S. L., Weeks O. B., Strang R.H.C., Thirkell D. Nature 214, 379, 1967

Joyce G. H., Hammond R. K., White D. C. J. Bacteriol 104, 323, 1970

Kaneda T. Can. J. Microbiol. 17, 169, 1971

Karrer P., Jucker E., "Carotenoide", Birkhauser, Basle 1948. Translated by Brand E. A. Elsevier, New York 1950.

Karrer P., Leumann E. Helv. Chim. Acta 34, 445, 1951

Kates M. Adv. Lipid Res. 2, 17, 1964

Kleinig H., Reichenbach H., Achenbach H. Arch. Microbiol. 74, 223, 1970

Kleinig H., Reichenbach H., Achenbach H., Stadler J. Arch. Microbiol. 78, 224, 1971

Kocur M. Private Communication 1970

Kocur M., Martinec T. Folia Prirod, Fakulty U.J.E.P. 3, 1, 1962

Kodicek E. In "Recent Progress in Microbiology" Intern. 8th Congr. Microbiol. Montreal 1952. University of Toronto Press, Montreal, 1965 P. 23

Krinsky, N. I. In "Carotenoids" edited by Isler O., Gutmann H., Solms U. Berkhauser Verlag Basel, P 669, 1971

Kuhn R., Sorensen N. A. Ber. Dt. Chem. Ges. 71, 1879, 1938

Kunisawa R., Stanier R. Y. Arch. Microbiol. 31, 146, 1958

Lennarz W. J., Talamo B. J. Biol. Chem. 241, 2207, 1966

McCormick A., Liaaen- Jensen S. Acta Chem. Scand. 20, 1989, 1966

MacFarlane M. G. Biochem J. 79, 4P, 1961

Maddy A. H., Kelly P. G. F.E.B.S. Letters 8, 341, 1970

Mathews M. M. J. Bact. 85, 322, 1963

Mathews M. M.,Krinsky N. I. Photochem. Photobiol. 4, 813, 1965

Mathews M. M., Sistrom W. R. J. Bact. 78, 778, 1959a.

Mathews M. M., Sistrom W. R. Nature 184, 1892, 1959b

Mathews M. M., Sistrom W. R. Arch. Microbiol. 35, 139, 1960

Metcalfe L. D., Schmitz A. A. Anal. Chem. 33, 363, 1961

Minnikin D. E., Abdolrahimzadeh H., Baddiley J. Biochem. Biophys. Acta 249, 651, 1971

Moore S., Stein W. H. J. Biol. Chem. 211, 907, 1954

Morrison S. J., Tornabene T. G., Kloos W. E. J. Bact. 108, 353, 1971

Norgard S., Francis G., Jensen A., Liaaen-Jensen S. Acta Chem. Scand. 24, 1460, 1970

O'Leary W. M. Bact. Revs. 26, 421, 1962

O'Leary W. M. "The Chemistry and Metabolism of Microbial Lipids" The World Publishing Co. Cleveland and New York, 1967.

Panos C., Cohen M. Biochim. Biophys. Acta 117, 98, 1966

Patch C. T., Landman O. E. J. Bact. 107, 345, 1971

Petracek F. J., Zechmeister L. Anal. Chem. 28, 1484, 1956

Prebble J. J. Gen. Microbiol. 52, 15, 1968

Ray P. H., White D. C., Brock T. D. J. Bact. 106, 25, 1971a

Ray P. H., White D. C., Brock T. D. J. Bact. 108, 227, 1971b

Razin S., Rottem S. J. Bact. 93, 1181, 1967

Reader V. Biochem J. 19, 1039, 1925

Reichenbach H., Kleinig H Arch. Microbiol. 76, 364, 1971

Rosypal S., Rosypalova A., Horejs J. J. Gen. Microbiol. 44, 281, 1966

Roth M. M. J. Bact. 93, 506, 1967

Roth M. M., Krinsky N. I. Photochem. Photobiol. 11, 555, 1970a

Roth M. M., Krinsky N. I. Biochim. Biophys. Acta 203, 357, 1970b

Salton M.R.J. Trans. N.Y. Acad. Sci. 29, 764, 1967a

Salton M.R.J. Prot. Biol Fluids 15, 279, 1967b

Salton M.R.J. Ann. Rev. Microbiol. 21, 417, 1967c

Salton M.R.J., Ehtisham-ud-din A.F.M. Austr. J. Exp. Biol. Med. Sci. 43, 255, 1965a

Salton M.R.J., Freer J. H. Biochim. Biophys. Acta 107, 531, 1965b

Saperstein S., Starr M. P. Biochem. Biophys. Acta 16, 482, 1955

Schmidt K., Pfennig N., Liaaen-Jensen S. Arch. Microbiol. 52, 132, 1965

Schneider W. C. In "Methods in Enzymology" Academic Press (New York and London) 3, 680, 1957

Schwieter U., Bolliger H. R., Chopard-Dit-Jean L. H., Kofler M., Koenig A., Von Planta C., Rueegg R., Vetter W., Isler O. Chimia (Aarau) 19, 294, 1965

Shaw N. Bact. Revs. 34, 365, 1970

Shaw N., Baddiley J. Nature 217, 142, 1968

Shaw N., Stead D. J. Bact. 107, 130, 1971

Shockman G. D., Kolb J. J., Bakay B., Conover M. J., Toennies G. J. Bact. 85, 168, 1963

Smith P. F. J. Gen. Microbiol. 32, 307, 1963

Smith P. F. Lipids 4, 331, 1969

Sobin B., Stahly G. L. J. Bact. 44, 265, 1942

Stanier R. Y. In "The Photochemical Apparatus : Its Structure and Function", Brookhaven Symp. Biol. 11, 43, 1959

Stimson W. H. F.E.B.S. Letters 13, 17, 1971

Strang R.H.C. Ph.D. Thesis, University of St Andrews 1968

Strang R.H.C., Thirkell D. Phytochem. 8, 431, 1969

Sueoka N. J. Molec. Biol. 3, 31, 1961

Sweeley C. C., Bentley R., Makita M., Wells W. W. J. Am. Chem. Soc. 85, 2497, 1963

Tadayan R. A., Carroll K. K. Lipids 6, 820, 1971

Theodore T. S., Popkin T. J., Cole R. M. Prep. Biocnem. 1, 233, 1971

Thierry O. C., Cooney J. J. Can. J. Microbiol. 12, 691, 1966

Thirkell D. J. Gen. Microbiol 55, 337, 1969

Thirkell D., Hunter M.I.S. J. Gen. Microbiol. 58, 293, 1969

Thirkell D., Hunter M.I.S. J. Gen. Microbiol. 62, 125, 1970

Thirkell D., Strang, R.H.C., Carstairs E. A. Phytochemistry 4, 155, 1965

Thirkell D., Strang, R.H.C., Chapman J. R. J. Gen. Microbiol. 49, 444, 1967

Toon P., Brown P. E., Baddiley J. Biochem J. 127, 399, 1972

Tornabene T. G., Bennett E. O., Oro J. J. Bact. 94, 344, 1967

Ueta N., Yanakawa T. Jap. J. Exp. Med. 38, 347, 1968

Ungers G. E., Cooney J. J. J. Bact. 96, 234, 1968

Veerkamp J. H. J. Bact. 108, 861, 1971

Vorbeck M. L., Marinetti G. V. Biochem. 4, 296, 1965a

Vorbeck M. L., Marinetti G. V. J. Lip. Res. 6, 3, 1965b

Ward J. B., Perkins H. R. Biochem J. 106, 391, 1968

Weedon B.C.L. In "Carotenoids" edited by Isler O, Gutmann H., Solms U. Berkhouser Verlag Basel, P 29, 1971

Weeks O. B., Andrewes A. G. Arch. Biochem. Biophys. 137, 284, 1970

Weibull C. J. Bact. 66, 688, 1953

Weibull C., Bergstrom L. Biochem. Biophys. Acta 30, 340, 1958

Wells M. A., Dittmer J. C., Biochemistry 2, 1259, 1963

Whistler R. L., Wolfrom M. L. "Methods in Carbohydrate Chemistry" Academic Press (New York and London) 1, 388, 1962

Work E. Nature 201, 1107, 1964

Work E., Griffiths H. J. Bact. 95, 641, 1968

Yamamoto H. Y., Chichester C. O., Nakayama T.O.M. Anal. Chem. 33, 1792, 1961

Yudkin M. D. Biochem J. 82, 40p, 1962

Yudkin M. D. Biochem J. 98, 923, 1966

Zechmeister L., Polgar A. J. Am. Chem. Soc. 65, 1522, 1943